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Detection of *Listeria monocytogenes* in artisanal soft cheeses from different street markets of Ecuador

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DEDICATORIA

"*Nuestra recompensa se encuentra en el esfuerzo y no en el resultado. Un esfuerzo total es una victoria completa*"
(Mahatma Gandhi)

A mis padres, por haber hecho de mi la persona que soy ahora. Mi respeto, cariño, admiración y gratitud para siempre.

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RESUMEN

L. monocytogenes es una de las bacterias más importantes relacionadas con las enfermedades transmitidas por los alimentos. Causa una enfermedad infecciosa conocida como listeriosis, que reporta un impacto significativo en la salud pública debido a su alta tasa de mortalidad (20-30% incluso con tratamiento). Los productos lácteos, como los quesos frescos, son un importante contribuyente a la infección por *L. monocytogenes;* y en el Ecuador, la mayoría de los hogares consumen quesos frescos. Este estudio tiene como objetivo determinar la prevalencia de *L. monocytogenes* en los quesos frescos artesanales, y comparar aislados de alimentos con aislados clínicos. Se colectaron un total de 260 muestras de queso fresco en 18 provincias de Ecuador y se analizaron 20 aislamientos clínicos obtenidos del Laboratorio de resistencia a los antibióticos del Instituto Nacional de Investigación de Salud Pública de Ecuador (INSPI). *Listeria* fue investigado por métodos bacteriológicos y enfoques moleculares. Encontramos que 37 (14.23%) muestras fueron positivas para *L. monocytogenes* en ambos métodos; el serotipo 4b se encontró en los aislamientos clínicos (75%) y en los alimentos (83,80%), seguido del serotipo 1/2b en los aislamientos clínicos (15%) y en los alimentos (8,10%) y el serotipo 1/2a en los aislamientos clínicos (10%) y en los alimentos (8.10%). Todos los aislamientos fueron susceptibles a los antibióticos. Por lo que sabemos, este es el primer reporte de los serotipos de *L. monocytogenes* asociados con alimentos y enfermedades clínicas en Ecuador.

Palabras clave: *Listeria monocytogenes,* queso fresco artesanal, serotipos, listeriosis, enfermedades trasmitidas por alimentos, y productos lácteos.

ABSTRACT

L. monocytogenes is one of the most important bacteria related with foodborne diseases. It causes an infectious disease known as listeriosis, which reports a significant impact on public health due to its high mortality rate (20-30% even with treatment). Dairy products such as soft cheese are an important contributor of *L. monocytogenes* infections; and in Ecuador, most households consume soft cheese. This study aims to determine the prevalence of *L. monocytogenes* in artisanal soft cheeses, and compare food and clinical isolates. A total of 260 fresh cheese samples were collected in 18 provinces of Ecuador, and 20 clinical isolates obtained from the Laboratory for Antibiotic Resistance from the National Institute of Public Health Research of Ecuador (INSPI) were analyzed. *Listeria* was investigated by bacteriological methods and molecular approaches. We found 37 (14.23%) samples were positive for *L. monocytogenes* in both methods; serotype 4b was found in clinical (75%) and food isolates (83.80%), followed by serotype 1/2b in clinical (15%) and food (8.10%) isolates and 1/2a in clinical (10%) and food (8.10%) isolates. All the isolates were susceptible to the antibiotics. To our knowledge, this is the first report of *L. monocytogenes* serotypes associated with food and clinical disease in Ecuador.

Key words: *Listeria monocytogenes,* artisanal soft cheeses, serotype, listeriosis, foodborne diseases, and dairy products.

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PART I: GENERAL INTRODUCTION

Foodborne diseases

Viruses, bacteria, parasites, toxins, metals, prions and other chemicals are related to the outbreaks of Foodborne Diseases (FBDs). The causes of many outbreaks reported in the Centers for Disease Control and Prevention (CDC) remain unknown, however virus and bacteria are the main pathogens associated with FBDs (Mead et al., 1999, McCabe-Sellers & Beattie, 2004).

The FBDs can be classified into five categories: infection, intoxication, metabolic disorders of food, allergies and idiosyncratic illnesses. Infections and poisonings can affect nearly everyone; yet some food-related diseases are classified as individualistic because they affect only few people in the population (Dodd et al., 2017).

Recently, FBDs incidence has increased considerably even though only 1 to 10% of the cases are included in official statistics (Díaz et al., 2013; Dodd et al., 2017). When sanitary guidelines are not implemented properly in the food industry, food can act as a disease transmission vehicle. The most common foodborne disease-causing pathogenic microorganisms are enterohaemorrhagic *Escherichia coli* (EHEC), *Salmonella sp*., *Vibrio cholerae*, *Staphylococcus aureus*, *Listeria monocytogenes,* etc. (Díaz et al., 2013; WHO, 2018).

Listeria genus

Listeria genus has shown to be ubiquitously distributed in a variety of environments such as land, waste and river water, affluent and even sewage treatment plants. In addition, it can be found in birds, fish, mollusks, crustaceans, insects, milk,

dairy and meat products, fruits and vegetables (Abrahão et al., 2008, González et al., 2009).

This genus consists of six species: *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. ivanovii*, *L. seeligeri* and *L. grayi*. Only *L. monocytogenes* and *L. ivanovii* are hemolytic species associated with human pathogenicity (Appendix 1.). Although the latter two species have differences in pathogenicity, their life cycle as an intracellular parasite is very similar (Bubert et al., 1999).

Both pathogenic species invade host cells, replicate in the cytosol after the phagosomal escape and spread from one cell to other cell by actin polymerization (Cocolin et al., 2002). This mechanism can be achieved by the presence of genetic determinants the *inl*AB operon, the LIPI-1 intracellular survival pathogenicity island and hpt intracellular growth locus in both species. Both species can infect different hosts: *L. monocytogenes* infect humans and ruminants, while *L. ivanovii*, only ruminants (Cocolin et al., 2002; Guillet et al., 2010; Toledo et al., 2018).

The ubiquitous nature of *Listeria spp.* allows it to contaminate food industry system and food chain. Human transmission of pathogenic strains of *Listeria spp*. can occur through the consumption of raw milk, dairy products or by ingestion of contaminated post-processed food (Albarracin et al., 2006). It is worth mentioning that as *L. innocua* is frequently reported in food, it can be an indicator of the presence of *L. monocytogenes*. It is known that both share the ecological niche, and that *L. innocua* has a competitive advantage due to its specific growth rate (Bubert et al., 1999; Albarracin et al., 2006; Gallegos et al., 2007).

Even though most outbreaks of human listeriosis and 85% of animal cases are caused by *L. monocytogenes*, there are rare cases reported of human infection with *L. ivanovii* in immune-compromised patients (Mazza et al., 2015). For instance, a case of

fatal bacteremia in a 62-year-old patient, caused by *L. innocua,* was reported in 2003, but the origin of this contamination could not be established (Perrin et al., 2003).

Listeria monocytogenes

Listeria monocytogenes, is a coccobacillus Gram-positive, facultative intracellular bacterium, resistant to different environmental conditions, tolerates a pH range between 4.4 to 9.6, high concentrations of sodium chloride (10%), temperature range of 1°C to 45°C, and it can support minimum water activity (aw) to grow (0.90) (Bubert et al., 1992; Baquero et al., 2006; Villalobos & E Martínez N, 2007; Abrahão et al., 2008; González et al., 2009; Muñoz, 2012).

It is widely distributed in nature: water, soil, vegetation and animal faeces. Domestic animals and wild mammals can be its reservoir. It has been isolated from various foods, especially raw products such as meats, milk and dairy products made with unpasteurized milk (Baquero et al., 2006; Muñoz, 2012). It adheres to food contact surfaces and forms biofilms, where microorganisms are embedded in an extracellular polymeric matrix protecting them from disinfectants and contamination is possible at any stage of the production chain. Thus, its control in food-processing facilities is complicated (Baquero et al., 2006; Muñoz, 2012).

L. monocytogenes virulence genes are organized into genetic units referred as pathogenicity islands, acquired by horizontal gene transfer. *L. monocytogenes* performs a coordinated regulation of the expression of its virulence genes (*plcA, hly, mpl, actA, plcB, inlA, inlB, inlC* and *hpt)* mainly by *prfA*, a protein that acts as an activator or repressor (Kathariou, 2002; López et al., 2006; Vera et al. al., 2013). Six of the virulence factor genes responsible for intracellular parasitism (*prfA, plcA, hly, mpl, actA* and *plcB*) are found in a region that encompasses 9 kb known as "*Listeria* pathogenicity island 1" (LIPI-1) or "virulence gene cluster" (vgc). Some of the key virulence factors

are hemolysin (listeriolysin O), and two phospholipases. Difference in virulence between the strains may be due to nucleotide polymorphisms caused by point mutations and/or the presence or absence of a virulence gene (Kathariou, 2002; López et al., 2006; Vera et al. al., 2013).

L. monocytogenes cause an infectious disease known as listeriosis, which can affect both humans and animals. The infection can appear in two forms: non-invasive listeriosis (or gastroenteritis), and invasive listeriosis (Aureli et al., 2000; Kathariou, 2002). The latter is the most serious one; it is characterized by fever, muscle pain and gastrointestinal symptoms, such as nausea or diarrhea. This infection can affect the nervous system, causing headache, confusion, and loss of balance or even seizures (Doumith et al., 2004; Abrahão et al., 2008). Pregnant women, newborns, older adults and immune-compromised individuals belong to the risk group of this disease. Although listeriosis is usually rare, it has a high case-fatality rate 20 to 30% (Aureli et al., 2000; Kathariou, 2002; Doumith et al., 2004; Abrahão et al., 2008; Datta et al., 2013).

L. monocytogenes has the ability to cross the three human physiological barriers: intestinal, hematoencephalic and placental (Camejo et al., 2011; Vera et al., 2013). After bacteria crosses the intestinal barrier, it is directed to the intestinal lumen where it traverses the epithelial cells, if the immune system cannot efficiently control the infection, it can proliferate and spread the infection to the bloodstream and to the lymph nodes. Once in the bloodstream, the bacteria reaches the liver and spleen, where it can replicate inside macrophages or epithelial cells (Camejo et al., 2011; Vera et al., 2013). If the replication of *L. monocytogenes* is not controlled by an innate immune response, the bacterium escapes and continues to replicate. The survival of the host depends ultimately on the development of an adaptive immune response. Otherwise, the bacteria

can reenter the bloodstream and reach brain or placenta, causing potentially lethal systemic infections (Camejo et al., 2011; Vera et al., 2013).

In spite the fact that, these bacteria have a remarkable ability to develop antibiotic resistance (Villalobos de Bastardo & Martínez Nazaret, 2006; Sosnowski et al., 2018. Studies confirm that the natural susceptibility of *L. monocytogenes* to the aminoglycosides, penicillins, quinolones, rifampicin, trimetropin associated with a sulfonamide (cotrimoxazole), fosfomycin and fusidic acid, is still maintained in the genus. However, some studies had demonstrated that there many strains resistant to oxacilin, ampicillin, streptomycin, erythromycin, vancomycin, chloramphenicol and tetracycline, has been detected in isolates from food and clinical infections (Villalobos de Bastardo & Martínez Nazaret, 2006; Sosnowski et al., 2018).

Serological classification of *Listeria monocytogenes*

Four serotypes have been identified according to their somatic antigen (O) (1/2, 3, 4 and 7), which combined with the flagellar antigens (H) make up thirteen serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 7). The group-specific somatic antigens (O-antigen) are referred to the wall teichoic acid (WTA) glycopolymers that represent the major antigenic determinants of *Listeria* cells (Vera et al., 2013; Eugster et al., 2015). The *Listeria* WTA polymer consists of a poly (ribitol-phosphate) backbone chain and is covalently attached to the peptidoglycan. The serovar diversity in *L. monocytogenes* is conferred by glycosidic substitutions on WTA units. Each serovar reveals point mutations in genes involved in WTA glycosylation (Callejo et al., 2008; Muñoz, 2012; Datta et al., 2013; Vera et al., 2013; Eugster et al., 2015). Although the teichoic acids of the cell wall seem to be of primary importance in determining the antigenicity, lipoteichoic acids present in *Listeria* strains must also be taken into

account when defining the biochemical bases of *Listeria* O antigenicity (Fiedler et al., 1984).

On the other hand, phylogenetic and subtyping studies, based on some virulence genes as *flaA*, *iap*, *hly* or *prfA*, have shown that *L. monocytogenes* is composed of three divergent lineages (I, II and III) (Rasmussen et al., 1995; Ward et al., 2004). Lineage I include serotypes 1/2b, 3b, 4b, 4d, 4e and 7, it has a greater pathogenic potential than Lineage II, composed of serotypes 1/2a, 1/2c, 3a and 3c. Lineage III contains three groups, IIIA, IIIB and IIIC, and the serotypes 4a, 4c and an atypical 4b (Fiedler et al., 1984; Callejo et al., 2008; Muñoz, 2012; Datta et al., 2013; Vera et al., 2013; Eugster et al., 2015). The size of the genome was determined to be very conserved and not correlated with a specific lineage (den Bakker et al., 2013). While a significant variation in size of the accessory genome was observed, its size is only a small percentage relative to the core genome. Therefore the size and the total number of genes are highly conserved across *L. monocytogenes* strains (den Bakker et al., 2013).

Of all the thirteen serotypes described, 1/2a, 1/2b and 4b are responsible for 95% of human listeriosis (both outbreaks and sporadic cases). Serotype 4b is the most frequent worldwide, and it is responsible of 50% of the large epidemic outbreaks reported, as well as 90 to 95% of sporadic cases. For these reasons, greater attention is being paid to *L. monocytogenes* serotype 4b (Kathariou, 2002; Doumith et al., 2004; Abrahão et al., 2008; Callejo et al., 2008; Muñoz, 2012; Burall et al., 2017).

It is commonly reported in ready-to-eat food (RTE), recently a variant strain of serotype 4b was detected in outbreaks (Datta & Burall, 2018). The new variant: 4bV contains an extra 6.3 kb DNA fragment. These new reported strains may represent the emergence of a group with altered virulence and/or environmental adaptation that

improves persistence and/or transfer (Kathariou, 2002; Burall et al., 2017; Datta & Burall, 2018).

Food associated with *Listeria*

Among all food products that serve as vehicles are RTE such as soft cheeses, dairy products, ice cream, pâtés, salads, delicatessen products, cold meats, smoked meats, sausages, smoked fish, seafood and vegetables are products from where *L. monocytogenes* has been isolated (Silva et al., 2014; Chen et al., 2016; Martín et al., 2016; Sosnowski et al., 2018).

Ready to eat products are consumed before being subjected to bactericidal processes and represent the largest risks (Chen et al., 2016; Martín et al., 2016); numerous outbreaks have been associated with consumption of soft cheeses, processed meats and processed seafood confirmed that food products are vehicles of transmission. Besides, previous studies have shown that cheese is a potential reservoir for *L. monocytogenes* because the greater availability of nutrients and the greater potential for contamination due to handling (Silva et al., 2014; Chen et al., 2016; Martín et al., 2016; Sosnowski et al., 2018).

Control of this pathogen in food-processing facilities is difficult as *L. monocytogenes* is able to withstand unsatisfactory pasteurization treatments, is capable to form biofilms and can easily adapt to the harsh environmental conditions (Espinoza M. et al., 2004; Ribeiro & Destro, 2014). Artisanal soft cheeses are one of the dairy products with greater health concern because they are made from unpasteurized milk, produced by inadequate manufacturing processes, are inappropriately stored for sale distribution, and have been reported to cause outbreaks of salmonellosis, listeriosis, and other enteric diseases (Espinoza M. et al., 2004; Martino et al., 2005; Villanueva Valencia, 2010; Arrese & Arroyo-Izaga, 2012; Díaz et al., 2013. The presence of *L.*

monocytogenes in soft cheeses has gained special importance lately as fatality rates in listeriosis outbreaks are approximately 30%, being one of the highest associated with foodborne diseases (Espinoza M. et al., 2004; Martino et al., 2005; Albarracin et al., 2006; González et al., 2009; Villanueva Valencia, 2010; Arrese & Arroyo-Izaga, 2012; Díaz et al., 2013).

Daily production of milk in Ecuador is around 3.5 million liters, of which, 31% is used for cheese production, 27% represents milk in plastic sheath; 20% milk in carton; 11% for milk powder; 10% for yogurt and 1% for other dairy products (Brassel & Hidalgo, 2007). In Ecuador, the northern (Carchi, Imbabura, Pichincha, Cotopaxi, Tungurahua, Bolívar, Chimborazo) and the southern Andean provinces (Cañar, Azuay and Loja) have a well develop artisanal and industrial dairy production (INEC, 2016; CIL, 2017). In terms of milk production, the Andean region contributes with 77.21% of the national production, followed by the Coast region with 17.96% and the Amazon region with 4.82% (Brassel & Hidalgo, 2007; Vizcarra, 2015; INEC, 2016; CIL, 2017).

The worldwide prevalence of *L. monocytogenes* in soft cheeses and dairy products is 2.2%. In European countries, it has been detected in percentages between 0 to 6%, but remarkably in Spain a frequency of 41% was reported (González et al., 2009). In Canada, USA and Mexico the prevalence vary between 0 and 12%. In South and Central America, in countries such as Cuba it was 5.6%; in Venezuela, 2%; in Bolivia, 17%; in Chile 22%. In Colombia, percentages have been found in a wide range: 78.3%, 15%, 29.6% and 33.1% and in Peru, percentages of 4.05% and 6.34% have been detected in fresh samples of artisanal cheese (da Silva et al., 1998; González et al., 2009; Díaz et al., 2013).

In Ecuador, there are few studies about *Listeria monocytogenes* in food. These studies are limited to detect DNA *Listeria* spp. by PCR in soft cheese, sausages, yogurt

and surfaces of the production plants. The main findings of these studies indicate the presence of *L. monocytogenes* in 9% of sausages and 4% of yogurts analyzed, also 80% of the cheese production plants in Riobamba presented *Listeria* spp., 55% of the cheeses from Guayaquil were related with the presence of *Listeria* spp. and 1 positive sample for *Listeria* spp. in a raw milk study in Pichincha (Palacios Mena, 2010; Castillo Segovia, 2013; Plaza Ibarra, 2013; Cabrera Rodríguez & Valladares Torres, 2016). However, the information in this topic is limited and not updated.

The presence of *L. monocytogenes* in cheeses, points to the occurrence of failure in the production process. The presence of other *Listeria spp*. indicates that the production process allows further contamination, which serves as an alert for a critical review of the process (Kabuki et al., 2004; Abrahão et al., 2008; Silva et al., 2014; Jackson et al., 2018).

Listeriosis in Ecuador

Listeriosis, unlike other foodborne infections, is characterized by a long incubation period, which makes it difficult to establish the relationship between the clinical case and the source of the infection. Although few cases of invasive listeriosis are reported, it has a significant impact on public health due to its high hospitalization rate (> 95%), high mortality rate (15-20% even with treatment) and morbidity in the long term for its association with devastating central nervous system syndromes (Callejo et al., 2008; Datta et al., 2013; Burall et al., 2017).

Human listeriosis affects mainly children, elder individuals, pregnant women and immune-compromised persons. Typical serious clinical manifestations of listeriosis are meningitis and/or encephalitis, abortions or neonatal infections, and septicemia. It is known that the infectious dose of *L. monocytogenes* is at least 10^2 viable cells in the risk group, this number increases to 10^4 in healthy population. This evidence supports

that all isolates of *L. monocytogenes* can be considered equally pathogenic; however there are several studies that indicate that virulence varies from strains to strain (López et al., 2006).

There is just one report of a clinical case related with listeriosis in Ecuador. It was a male neonatal with signs characterized by respiratory failure and coetaneous lesions by which he was considered an infected neonate. After all the investigation the causal pathogen was determined as *L. monocytogenes*, the identification was made by culture, but the origin of the infection remained unknown (Kittyle et al., 2009).

PART II: SCIENTIFIC ARTICLE

Detection of *Listeria monocytogenes* in artisanal soft cheeses from different street markets of Ecuador

Introduction

Foodborne diseases (FBDs) are a problem for every country in the world. Estimates of the overall number of episodes of FBDs are helpful for allocating resources and prioritizing interventions (Scallan et al., 2011). Despite strict controls that are imposed to the food industry, each year approximately three million people die because of FBDs (EFSA & ECDC, 2016; CDC, 2018; Soto-Varela et al., 2018).

L. monocytogenes cause an infectious disease known as listeriosis, which can affect both humans and animals. The infection can appear in two forms: non-invasive gastroenteritis, and invasive listeriosis. Pregnant women, newborns, older adults and immune-compromised individuals belong to the risk group of this disease. Although few cases of invasive listeriosis are reported, it has a significant impact on public health due to its high hospitalization rate (> 95%), high mortality rate (15-20% even with treatment) and morbidity in the long term for its association with devastating central nervous system syndromes (Aureli et al., 2000; Kathariou, 2002; Doumith et al., 2004; Abrahão et al., 2008; Callejo et al., 2008; Datta et al., 2013; Burall et al., 2017).

Listeria monocytogenes is often found in the microbiota of ruminants, and represents a serious health hazard for the community with dairy and other farm products being the most important vehicles for the transmission of infection (Bandelj et al., 2018). The contamination of some varieties of cheeses with *Listeria monocytogenes* is an important problem for public health, as well as for the industrial financial losses

(Dalzini et al., 2017; Melero et al., 2018). This bacteria has the ability to prosper in different environmental conditions such as pH (4.4 to 9.6), high concentrations of sodium chloride, and growth temperature range of 1 to 45°C (Bubert et al., 1992; Baquero et al., 2006; Villalobos & E Martínez N, 2007; Abrahão et al., 2008; González et al., 2009; Muñoz, 2012).

Among all food products that serve as pathogenic vehicles, RTE such as soft cheeses, dairy products, ice cream, pâtés, salads, delicatessen products, cold meats, smoked meats, sausages, smoked fish, seafood and vegetables are products from where *L. monocytogenes* has been isolated (Silva et al., 2014; Chen et al., 2016; Martín et al., 2016; Sosnowski et al., 2018). Food items from the processes dairy food category such as soft cheese are an important contributor of *L. monocytogenes* infections (Jagadeesan et al., 2018). Artisanal cheeses made with unpasteurized milk are a potential vehicle of transmission of pathogenic microorganisms (Espinoza M. et al., 2004; Martino et al., 2005; Albarracin et al., 2006; González et al., 2009; Villanueva Valencia, 2010; Arrese & Arroyo-Izaga, 2012; Díaz et al., 2013).

The worldwide prevalence of *L. monocytogenes* in soft cheeses and dairy products is 2.2%; in Canada, USA and Mexico the prevalence vary between 0 and 12%; in South and Central America (including Cuba) 5.6%; in Venezuela, 2%; in Bolivia, 17%; in Chile 22%. In Colombia, percentages have been found in a wide range: 78.3%, 15%, 29.6% and 33.1% and in Peru, percentages of 4.05% and 6.34% have been detected in fresh samples of artisanal cheese (da Silva et al., 1998; González et al., 2009; Díaz et al., 2013).

Thirteen serotypes had been described, but only three of them, 1/2a, 1/2b and 4b are involved in 95% of human listeriosis (both outbreaks and sporadic cases). Serotype 4b is the most frequent worldwide, being responsible of 50% of the large epidemic

outbreaks reported, as well as 90 to 95% of sporadic cases (Kathariou, 2002; Doumith et al., 2004; Abrahão et al., 2008; Callejo et al., 2008; Muñoz, 2012; Burall et al., 2017).

The impact of human listeriosis in Ecuador is unknown therefore this study aimed to determine the prevalence of *L. monocytogenes* in soft cheeses and investigate the presence of serotypes associated with human disease in these products.

Materials and methods

Sample collection.

A total of 260 samples of artisanal soft cheeses (lacking a sanitary registration certificate issued by the National Authority) were collected in 18 provinces of Ecuador. To evaluate the number of samples, the sample size for a single proportion formula were used, with a 95% of confidence and 5% of precision (Appendix 2).

$$n = \frac{(Z_\alpha)^2 \times p \times q}{d^2}$$

$$(Z_\alpha)^2 = Constant\ (3.842)\ with\ 95\%\ of\ confidence$$

$$p = percentaje\ obtained\ from\ other\ studies$$

$$q = 1 - p$$

$$d^2 = 5\%\ of\ precision$$

Isolation of pure culture of L. monocytogenes.

All the 260 samples analyzed were cultured in the esculin based Palcam *Listeria* selective Agar (BD^{TM}) for 24 hours at 37 ºC, for the isolation and detection of *Listeria monocytogenes* and other *Listeria* species.

Typical *Listeria* species colonies are approximately 1 mm diameter, and present a gray to black colonies surrounded by a dark brown to black halo) (FDA, 2017). Two

isolates per sample were recovered and stored at -80 ºC in Skim milk with 10% glycerol for further molecular analysis.

DNA extraction and molecular analysis.

DNA extraction was performed, using DNAzol (Invitrogen™ Carlsbad, USA) from all the isolates of *L. monocytogenes* and *Listeria* spp. obtained from the samples. Additionally, we extracted DNA (using Pure Link™ Invitrogen Kit Carlsbad, USA) from 20 clinical isolates from Laboratory for Antibiotic Resistance from the National Institute of Public Health Research of Ecuador (INSPI), collected since June 2015 until July 2018) and isolated by the were analyzed.

PCR reactions to determine the different *Listeria* species and serotypes of *L. monocytogenes* were carried out following the standardized protocols established by Tao et al., 2016 and Doumith et al., 2004, respectively. As a positive control for both analysis, strain *L. monocytogenes* 4b ATCC 13932 was used.

We used universal primers for the bacterial 16S ribosomal to confirm the *Listeria* species PCR. The primers used were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3').

Molecular detection of L. monocytogenes.

Following the manufacturer's instructions of the Molecular Detection System (MDS 3M™), twenty-five grams (g) of each sample were pre-enriched with 225 ml of demi-fraser broth with ferric ammonium citrate. After pre-enrichment, samples were examined with the MDS kit for *L. monocytogenes* (3M™).

Results

After the analysis of the 260 soft cheese samples. After the analysis of the 260 soft cheese samples. Thirty-seven were positive for *Listeria monocytogenes,* those results were confirmed by bacteriological and molecular analyses.

The 37 positive samples for *Listeria monocytogenes* in soft cheeses represented 14.23%, 95% CI (10.2-19.1) of prevalence of this specie in artisanal cheese (Figure 1).

From 37 food isolates, 31 (83.80%) belonged to serotype 4b, 8.10% (3) to serotype 1/2a, and 8.10% (3) to serotype 1/2b. While from clinical isolates, 75% (15) belonged to serotype 4b, 10% (2) to the serotype 1/2a, and 15% (3) to the serotype 1/2b (Table 1).

All strains were sensitive to trimethropin/sulphamethoxazole, penicillin, ampicillin, erythromycin and meropenem.

Ten additional samples were positive for *Listeria spp*., those results were obtained by bacteriological analysis. The targets for the detection and differentiation of *L. monocytogenes, L. ivanovii* and other non-pathogenic *Listeria* spp., used were: LMOf2365_2721 encoding a glycosyl hydrolase, AX25_00730 encoding a transcriptional regulator, lin1814 encoding a hypothetical protein, *int* encoding a phage integrase and lwe1673 encoding a conserved hypothetical protein (Tao et al., 2016). Those extra positive samples were confirmed by PCR and belonged to *L. innocua* (88.88%), *L. welshimeri* (5.6%) and *L. grayi* (5.6%). Nine of the 37 samples presented both *L. monocytogenes* and *L. innocua* in the same sample (24.32%).

Neither *L. ivanovii* nor *L. seeligeri* were found in the samples analyzed in the present study.

The sequences obtained with the 16S rDNA amplification are available on the Genbank database with the accession number MK235117 for *L. monocytogenes* and MK235116 for *L. innocua.*

Discussion

We found that 83.80% cheese samples contained *L. monocytogenes* serotype 4b (lineage I); the same serotype was found in 75% of clinical isolates. Other serotypes found were 1/2b 6 (10.52%), and 4 were 1/2a (7.02%) (Table 1). Several studies have concluded that sporadic cases and food isolations are commonly associated with serotypes 4b, 1/2a and 1/2b. These three serotypes are considered to be the most pathogenic and are responsible for more than 95% of invasive listeriosis cases (Orsi et al., 2011; Nastasijevic et al., 2017; Zoz et al., 2017; López et al., 2006; Artursson et al., 2018).

The high percentage of serotype 4b of this study (73.68%) is of great importance, because this serotype is responsible for 50 and 70% of the clinical cases and outbreaks worldwide (Kathariou, 2002; López et al., 2006; Muñoz, 2012). The presence of this serotype in soft cheese in Ecuador because people eat this cheese without any additional cooking. *L. monocytogenes* serotype 4b strains replicate more than the other serotypes in monocytes/macrophages, this feature may be involved in their pathogenicity, particularly in the growth and dissemination of the bacteria through the host body (Hasebe et al., 2017).

It is known that *L. monocytogenes* can coexist with competing microorganisms and other *Listeria spp*. strains (Gallegos et al., 2007). Other *Listeria* species were isolated in this study. The most important is the percentage recovery of *L. innocua* in (88.88%), this is the phylogenetically closest species to *L. monocytogenes,* sharing the

ecological niche (Curiale & Lewus, 1994; Albarracin et al., 2006; Tao et al., 2016; Gallegos et al., 2007).

L. innocua is a non-pathogenic bacteria, its frequent presence in food suggests the possibility of *L. monocytogenes* growth, which represents a potential risk for consumers (Bubert et al., 1999). The presence of other *Listeria spp*. such as *L. welshimeri* and *L. grayi*, found in cheeses, may be associated with inadequate practices of processing, storage and transportation of this product (Cocolin et al., 2002; Abrahão et al., 2008; González et al., 2009).

In recent years, listeriosis has become one of the most important foodborne diseases, with a high relevance for public health due to the severity of the symptoms, the high rate of hospitalization and mortality (Medrano et al., 2006; Nastasijevic et al., 2017). In Ecuador, the impact of listeriosis on public health is unknown because it is not a notifiable disease. To our knowledge, this study is the first of its kind in Ecuador determining the serotype of *Listeria monocytogenes* in food and clinical samples, also demonstrating by culture that the cells present in food were viable. Investigations of listeriosis outbreaks often involve processed and ready-to-eat meats, dairy products and raw products (Heiman et al., 2016). Regulatory initiatives and industry actions implemented between 1998 and 2008 have reduced outbreaks from ready-to-eat (RTE) red meats and poultry. In contrast, listeriosis outbreaks from dairy products showed no decrease in frequency (Cartwright et al., 2013). Thus soft cheese was an adequate sample to look for this bacterium.

Soft cheese provides perfect conditions for *Listeria* growth Callejo et al., 2008; Silva et al., 2014).

In this study, *L. monocytogenes* was found in 14.23% of food sampled in 2018, and in 20 clinical samples received from INSPI. These results are comparable with

similar studies carried out in different Latin American countries such as Costa Rica 10% (Ellner et al., 1991), Colombia 13.3% (Baquero et al., 2006), Bolivia 17% (Díaz et al., 2013), Cuba 1.9% (Martino Zagovalov et al., 2005), Venezuela 17.5% (González et al., 2009), Peru 17.3% (González et al., 2009), Brazil 8% (Cavalet et al., 2002) and Chile 15% (Villanueva & Salazar, 2017).

The presence of *L. monocytogenes* in food samples suggests that there may be sporadic cases or outbreaks of listeriosis in Ecuador, however there is no monitoring of this pathogen and therefore lack of epidemiological data. On the other hand, presence of *L. monocytogenes* and in general *Listeria* spp. in cheeses, suggests the absence of an adequate heat treatment (pasteurization), contamination after process and production, and presence of biofilms in the processing plant (da Silva et al., 1998).

All the clinical and food isolates analyzed in this study, determined that all the strains from 2015 to 2018, are still susceptible to the antimicrobial agents used in the treatment of listeriosis. These results agreed with a retrospective analysis made in Argentina, in which strains from a 20-year period have not changed their susceptibility pattern (Prieto et al., 2016), and with studies made in Europe, the United States and Latin America (Safdar & Armstrong, 2003, Hansen et al., 2005, Reis et al., 2011, Ruiz-Bolivar et al., 2011, Gamboa-Marín et al., 2013, Kuch et al., 2018). However, additional studies have reported isolates with high levels of multiresistance to some antibiotics (Morvan et al., 2010, Escolar et al., 2017, Tang et al., 2017, Sharma et al., 2017, Noll et al., 2018; Sosnowski et al., 2018).

To date, there is a lack of knowledge about the genomic diversity of *L. monocytogenes* isolates in Ecuador from both humans and food. Genomic epidemiology studies of *L. monocytogenes* are necessary to assess the relationships between strains, and identify genetically related isolates and clones associated with highly abundant

outbreaks (Chen et al., 2007; Bergholz et al., 2018; Muhterem-Uyar et al., 2018). Sub-typing of *L. monocytogenes* plays an important role in epidemiology during outbreak and tracking investigations (Datta et al., 2013).

In conclusion, this study provides a baseline for further research of food and positive clinical cases for *L. monocytogenes* in Ecuador. Our data suggest that *L. monocytogenes* could be serious public health problem, which hasn't been detected in the past. There should be continuous *L. monocytogenes* monitoring of cheeses and other dairy products in Ecuador. A timely identification of the source of contamination would help to trace the origin and minimize exposure, implementing better control strategies to prevent foodborne listeriosis.

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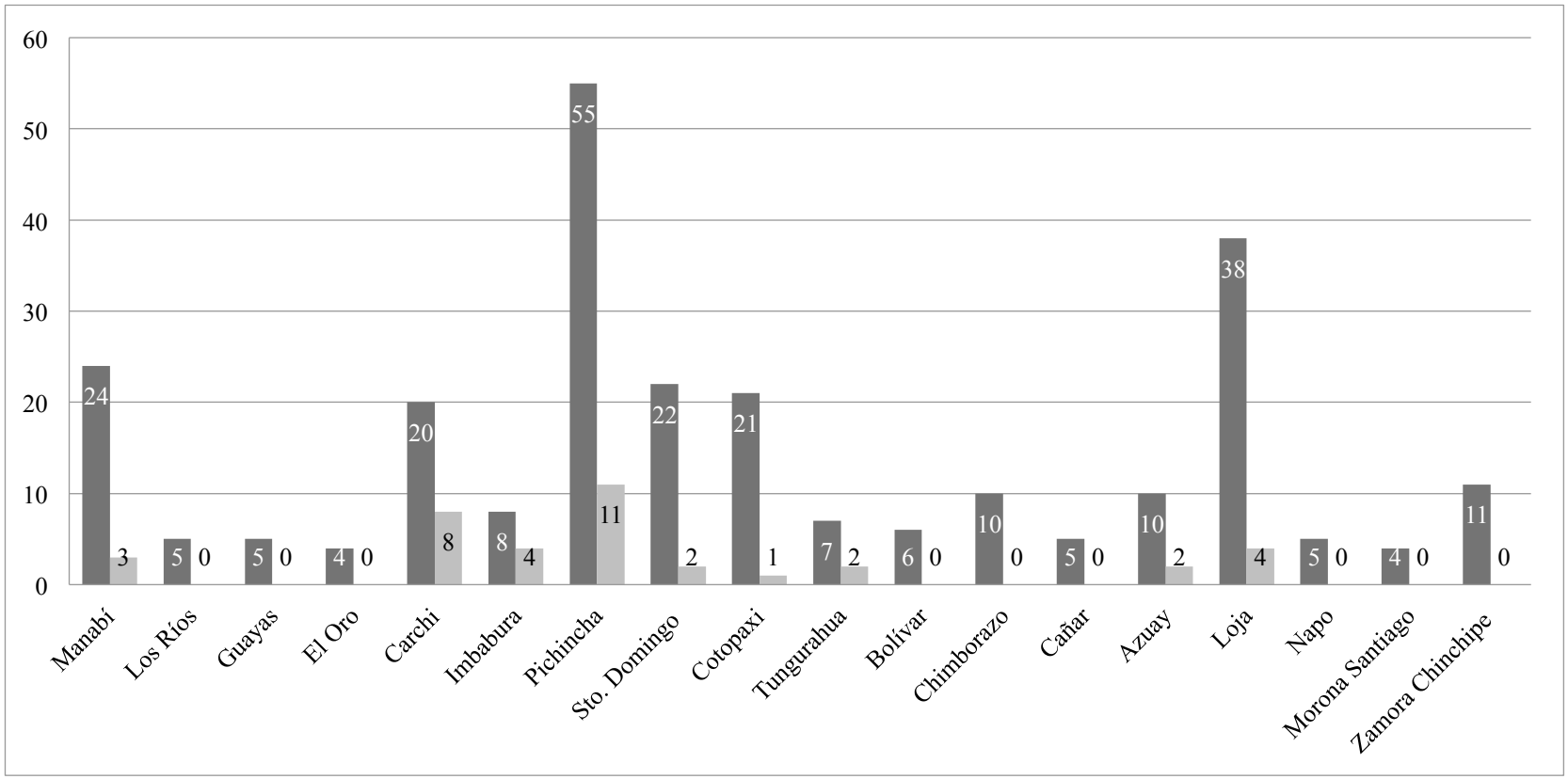
FIGURES

Figure 1. **Distribution of samples analyzed from 18 provinces of Ecuador.**



The total number of samples analyzed from each province is represented in black columns; positive samples are represented in gray columns.

TABLES

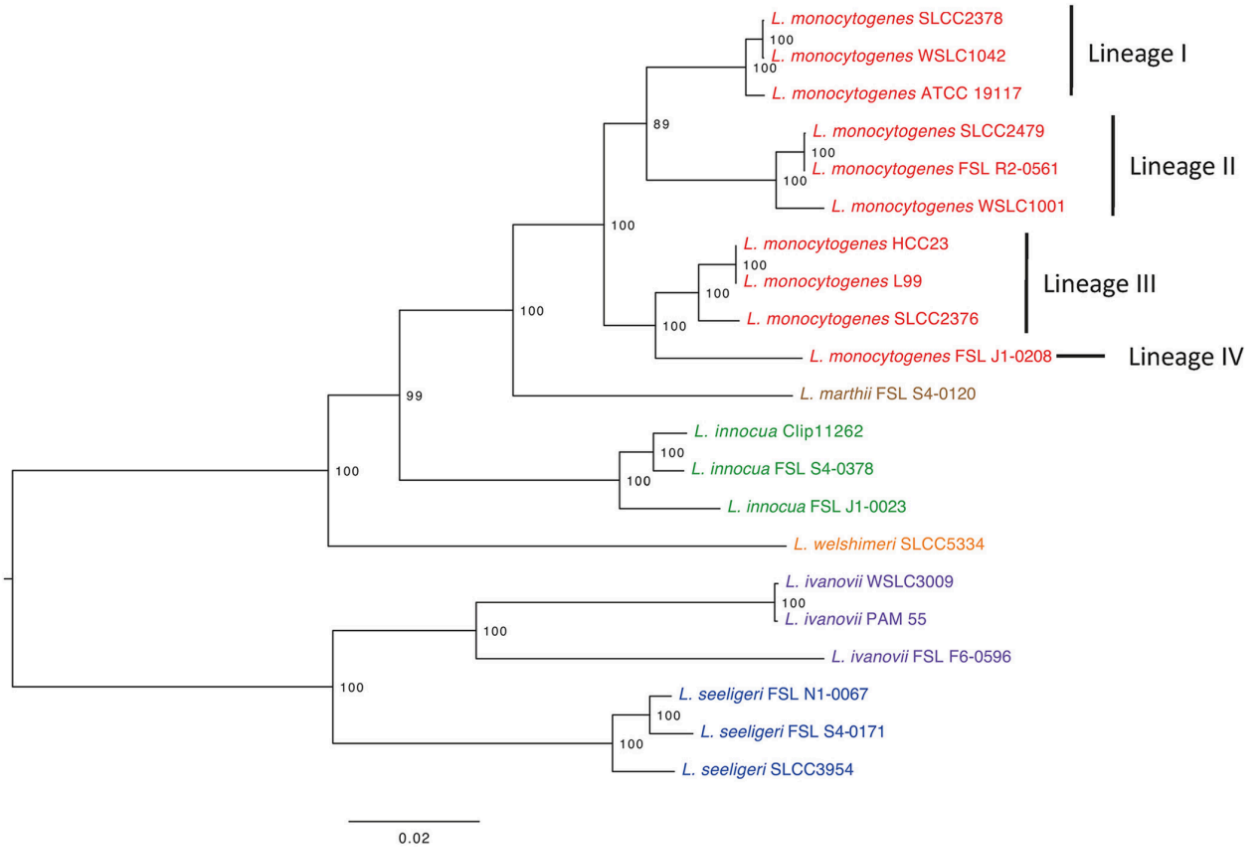
Table 1. **Distribution of positive samples for *Listeria monocytogenes* and serotypes.**

Sample Number	Date of collection	Origin of sample	Serotype
1	June 2015	Clinical	1/2a
2	January 2016	Clinical	4b
3	March 2016	Clinical	4b
4	April 2016	Clinical	4b
5	September 2016	Clinical	4b
6	January 2017	Clinical	4b
7	January 2017	Clinical	4b
8	April 2017	Clinical	4b
9	May 2017.	Clinical	1/2b
10	October 2017	Clinical	1/2b
11	November 2017	Clinical	4b
12	March 2018	Clinical	4b
13	April 2018	Clinical	4b
14	April 2018	Clinical	1/2b
15	April 2018	Clinical	4b
16	May 2018.	Clinical	1/2a
17	May 2018.	Clinical	4b
18	June 2018	Clinical	4b
19	July 2018	Clinical	4b
20	July 2018	Clinical	4b
21	March 2018	Soft cheese	4b
22	March 2018	Soft cheese	4b
23	March 2018	Soft cheese	1/2 b
24	March 2018	Soft cheese	1/2 a
25	March 2018	Soft cheese	1/2 b
26	March 2018	Soft cheese	4b
27	March 2018	Soft cheese	4b
28	March 2018	Soft cheese	4b
29	March 2018	Soft cheese	4b
30	March 2018	Soft cheese	1/2 a
31	March 2018	Soft cheese	4b
32	March 2018	Soft cheese	4b
33	March 2018	Soft cheese	1/2 b
34	March 2018	Soft cheese	4b
35	April 2018	Soft cheese	4b

Sample Number	Date of collection	Origin of sample	Serotype
36	April 2018	Soft cheese	4b
37	April 2018	Soft cheese	4b
38	April 2018	Soft cheese	4b
39	April 2018	Soft cheese	1/2 a
40	April 2018	Soft cheese	4b
41	May 2018	Soft cheese	4b
42	May 2018	Soft cheese	4b
43	May 2018	Soft cheese	4b
44	May 2018	Soft cheese	4b
45	May 2018	Soft cheese	4b
46	May 2018	Soft cheese	4b
47	June 2018	Soft cheese	4b
48	July 2018	Soft cheese	4b
49	July 2018	Soft cheese	4b
50	July 2018	Soft cheese	4b
51	July 2018	Soft cheese	4b
52	August 2018	Soft cheese	4b
53	August 2018	Soft cheese	4b
54	August 2018	Soft cheese	4b
55	September 2018	Soft cheese	4b
56	September 2018	Soft cheese	4b
57	September 2018	Soft cheese	4b

APPENDIX

Appendix 1. **Phylogenetic tree of *Listeria* species.**
Taken from Liao et al., 2017. Phylogenetic tree inferred by maximum likelihood method using the core genome SNPs of 21 *Listeria* sensu stricto isolates. kSNP2 with kmer size of 19 was used to identify core genome SNPs that were used for constructing the tree with the GTRG substitution model and 1,000 bootstrap repetitions. The tree is rooted by the midpoint. Only bootstrap values of >70% are presented on the tree. *L. monocytogenes* is indicated in red, *L. marthii* is in brown, *L. innocua* is in green, *L. welshimeri* is in yellow, *L. seeligeri* is in blue, and *L. ivanovii* is in purple.



https://aem.asm.org/content/aem/83/12/e00306-17.full.pdf

Appendix 2. **Sample size for a single proportion**

The values used in this analysis was based on studies form other countries of the region (Brazil 8% (Cavalet et al., 2002) and Peru 17.3% (González et al., 2009)).

$$n = \frac{(Z_\alpha)^2 \times p \times q}{d^2}$$

Brazil 8%:

$$n = \frac{(3.842)(0.08)(0.92)}{(0.05)^2} = \frac{0.2827}{0.0025} = 113.10$$

Perú 17.3% :

$$n = \frac{(3.842)(0.173)(0.827)}{(0.05)^2} = \frac{0.5496}{0.0025} = 219.87$$

Appendix 3. **Description of collected samples**

Site of collection of the 260 fresh samples of artisanal soft cheeses.

Sample ID	Province
Lm 01	Pichincha
Lm 02	Pichincha
Lm 03	Pichincha
Lm 04	Cotopaxi
Lm 05	Cotopaxi
Lm 06	Pichincha
Lm 07	Imbabura
Lm 08	Carchi
Lm 09	Carchi
Lm 10	Imbabura
Lm 11	Carchi
Lm 12	Carchi
Lm 13	Carchi
Lm 14	Carchi
Lm 15	Carchi
Lm 16	Carchi
Lm 17	Carchi
Lm 18	Pichincha
Lm 19	Manabi
Lm 20	Carchi
Lm 21	Carchi
Lm 22	Pichincha
Lm 23	Carchi
Lm 24	Carchi
Lm 25	Pichincha
Lm 26	Carchi
Lm 27	Manabi
Lm 28	Carchi
Lm 29	Carchi
Lm 30	Carchi
Lm 31	Manabi
Lm 32	Manabi
Lm 33	Pichincha
Lm 34	Pichincha
Lm 35	Carchi
Lm 36	Manabi
Lm 37	Manabi
Lm 38	Carchi
Lm 39	Imbabura
Lm 40	Carchi
Lm 41	Pichincha

Sample ID	Province
Lm 42	Pichincha
Lm 43	Pichincha
Lm 44	Pichincha
Lm 45	Pichincha
Lm 46	Pichincha
Lm 47	Pichincha
Lm 48	Cotopaxi
Lm 49	Cotopaxi
Lm 50	Cotopaxi
Lm 51	Manabi
Lm 52	Cotopaxi
Lm 53	Cotopaxi
Lm 54	Cotopaxi
Lm 55	Cotopaxi
Lm 56	Cotopaxi
Lm 57	Cotopaxi
Lm 58	El Oro
Lm 59	Chimborazo
Lm 60	Tungurahua
Lm 61	Chimborazo
Lm 62	Chimborazo
Lm 63	Bolivar
Lm 64	Tungurahua
Lm 65	Tungurahua
Lm 66	Cotopaxi
Lm 67	Tungurahua
Lm 68	Morona Santiago
Lm 69	Cotopaxi
Lm 70	Cotopaxi
Lm 71	Cotopaxi
Lm 72	Tungurahua
Lm 73	Tungurahua
Lm 74	Cotopaxi
Lm 75	Pichincha
Lm 76	Pichincha
Lm 77	Cotopaxi
Lm 78	Cotopaxi
Lm 79	Cotopaxi
Lm 80	Pichincha
Lm 81	Chimborazo
Lm 82	Chimborazo
Lm 83	Chimborazo
Lm 84	Chimborazo
Lm 85	Chimborazo
Lm 86	Chimborazo

Sample ID	Province
Lm 87	Chimborazo
Lm 88	Cañar
Lm 89	Cañar
Lm 90	Cañar
Lm 91	Cañar
Lm 92	Loja
Lm 93	Loja
Lm 94	Loja
Lm 95	Loja
Lm 96	Loja
Lm 97	Loja
Lm 98	Loja
Lm 99	Loja
Lm 100	Zamora Chinchipe
Lm 101	Zamora Chinchipe
Lm 102	Morona Santiago
Lm 103	Zamora Chinchipe
Lm 104	Morona Santiago
Lm 105	Zamora Chinchipe
Lm 106	Zamora Chinchipe
Lm 107	Zamora Chinchipe
Lm 108	Zamora Chinchipe
Lm 109	Zamora Chinchipe
Lm 110	Zamora Chinchipe
Lm 111	Zamora Chinchipe
Lm 112	Zamora Chinchipe
Lm 113	Loja
Lm 114	Loja
Lm 115	Loja
Lm 116	Loja
Lm 117	Loja
Lm 118	Loja
Lm 119	Loja
Lm 120	Loja
Lm 121	Loja
Lm 122	Loja
Lm 123	Loja
Lm 124	Loja
Lm 125	Loja
Lm 126	Loja
Lm 127	Loja
Lm 128	Loja
Lm 129	Loja
Lm 130	Loja
Lm 131	Loja

Sample ID	Province
Lm 132	Loja
Lm 133	Loja
Lm 134	Loja
Lm 135	Loja
Lm 136	Loja
Lm 137	Loja
Lm 138	Loja
Lm 139	Loja
Lm 140	Loja
Lm 141	Loja
Lm 142	Azuay
Lm 143	Azuay
Lm 144	Azuay
Lm 145	Azuay
Lm 146	Azuay
Lm 147	Azuay
Lm 148	Azuay
Lm 149	Azuay
Lm 150	Azuay
Lm 151	Pichincha
Lm 152	Pichincha
Lm 153	Pichincha
Lm 154	Pichincha
Lm 155	Pichincha
Lm 156	Cotopaxi
Lm 157	Manabi
Lm 158	Pichincha
Lm 159	Pichincha
Lm 160	Pichincha
Lm 161	Manabi
Lm 162	Pichincha
Lm 163	Pichincha
Lm 164	Pichincha
Lm 165	Pichincha
Lm 166	Pichincha
Lm 167	Pichincha
Lm 168	Pichincha
Lm 169	Pichincha
Lm 170	Pichincha
Lm 171	Cotopaxi
Lm 172	Pichincha
Lm 173	Pichincha
Lm 174	Pichincha
Lm 175	Pichincha
Lm 176	Pichincha

Sample ID	Province
Lm 177	Pichincha
Lm 178	Pichincha
Lm 179	Pichincha
Lm 180	Pichincha
Lm 181	Pichincha
Lm 182	Pichincha
Lm 183	Pichincha
Lm 184	Pichincha
Lm 185	Pichincha
Lm 186	Santo Domingo
Lm 187	Pichincha
Lm 188	Santo Domingo
Lm 189	Santo Domingo
Lm 190	Santo Domingo
Lm 191	Los Ríos
Lm 192	Santo Domingo
Lm 193	Santo Domingo
Lm 194	Manabi
Lm 195	Santo Domingo
Lm 196	Manabi
Lm 197	Santo Domingo
Lm 198	Santo Domingo
Lm 199	Santo Domingo
Lm 200	Los Ríos
Lm 201	Manabi
Lm 202	Santo Domingo
Lm 203	Santo Domingo
Lm 204	Santo Domingo
Lm 205	Santo Domingo
Lm 206	Santo Domingo
Lm 207	Manabi
Lm 208	Manabi
Lm 209	Manabi
Lm 210	Manabi
Lm 211	Santo Domingo
Lm 212	Santo Domingo
Lm 213	Santo Domingo
Lm 214	Manabi
Lm 215	Manabi
Lm 216	Manabi
Lm 217	Santo Domingo
Lm 218	Santo Domingo
Lm 219	Manabi
Lm 220	Pichincha
Lm 221	Santo Domingo

Sample ID	Province
Lm 222	Manabi
Lm 223	Manabi
Lm 224	Santo Domingo
Lm 225	Manabi
Lm 226	Loja
Lm 227	Bolivar
Lm 228	Bolivar
Lm 229	Bolivar
Lm 230	Bolivar
Lm 231	Bolivar
Lm 232	Guayas
Lm 233	Guayas
Lm 234	Guayas
Lm 235	Guayas
Lm 236	Guayas
Lm 237	Los Ríos
Lm 238	El Oro
Lm 239	Imbabura
Lm 240	El Oro
Lm 241	El Oro
Lm 242	Imbabura
Lm 243	Imbabura
Lm 244	Cañar
Lm 245	Napo
Lm 246	Napo
Lm 247	Napo
Lm 248	Napo
Lm 249	Los Ríos
Lm 250	Los Ríos
Lm 251	Tungurahua
Lm 252	Napo
Lm 253	Imbabura
Lm 254	Imbabura
Lm 255	Morona Santiago
Lm 256	Pichincha
Lm 257	Pichincha
Lm 258	Manabi
Lm 259	Azuay
Lm 260	Pichincha