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Presence of indicator bacteria, Shiga toxin-producing *Escherichia coli*, and nontuberculous mycobacteria in oregano

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Abstract

Limited data are available regarding microbial contamination in oregano. This study aimed to evaluate the microbiological quality and presence of diarrheagenic *Escherichia coli* pathotypes and nontuberculous mycobacteria (NTM) in 85 oregano samples purchased from different markets in Mexico City. All samples analyzed were positive for aerobic-mesophilic bacteria, with limits ranging from 1.14 to 6.5 log CFU/g. A total of 67, 41, and 9 samples were positive for total coliforms, fecal coliforms, and *E. coli*, respectively, present at concentrations ranging from <3 to >1,100 MPN/g. One sample harbored Shiga toxin-producing *E. coli* strains positive for the Shiga toxin 2 (*stx*2) locus at a concentration of 3 MPN/g. NTM species were recovered from 15 samples and included *M. fortuitum* (six), *M. smegmatis* (two), *M. conceptionense* (one), *M. porcinum* (one), *M. parafortuitum* (one), *M. flavescens* (one), *M. goodii* (one), and two strains that could not be identified. Measures to diminish the high levels of microorganisms and pathogenic bacteria in this food item might be advisable. Good hygiene practices, good manufacturing practices, and hazard analysis and critical control points must be applied throughout the chain of production to ensure the safety of oregano.

Keywords: oregano; microbiological quality; shiga toxin-producing *E. coli*; nontuberculous mycobacteria.

Practical Application: Oregano samples analyzed had unsatisfactory microbiological quality, and some harbored Shiga toxin-producing *E. coli* and nontuberculous mycobacteria strains associated with illness. Good hygiene practices, good manufacturing practices, and hazard analysis and critical control points are crucial and must be applied throughout the chain of production to ensure the safety of oregano.

1. INTRODUCTION

In Mexico, several species regarded as oregano are included in various genera of the *Lamiaceae* and *Verbenaceae* families, as well as a few species in the *Asteraceae* and *Fabaceae* families. Two popular Mexican oreganos are *Lippia graveolens* Kunth (Syn: L. berlandieri Schauer) and *Poliomintha longiflora* A. Gray (Rivero-Cruz et al., 2011). The oregano plant is a shrub that reaches up to 2.5 m in height and develops an average of 1.2 m of foliage. The plant has branched stems with a large number of leaves that constitute the usable part (Huerta, 1997). The commercial production of oregano in Mexico is concentrated mainly in the states of Durango, Guanajuato, Jalisco, Queretaro, San Luis Potosi, and Zacatecas (Huerta, 1997). Other states that have joined the production of this resource are Chihuahua, Coahuila, Oaxaca, Puebla, and Tamaulipas (Angulo et al., 2004). Mexico ranks second in the production of dry oregano, with around 4,000 tons per year (Gobierno de Mexico, 2013); 85% of this production is exported to the United States, 10% is sold in the national market, and the remaining 5% is exported to European and Asian countries (Orona et al., 2017). In Mexico, the fresh and dried leaves of the oregano plant are used as an antiasthmatic, antispasmodic, antitussive, anthelmintic, and fungicide, as well as for the treatment of menstrual disorders and diabetes (Huerta, 1997; Pascual et al., 2001). In oregano samples, the presence of *Bacillus cereus*, bacteria belonging to the *Enterobacteriaceae* family, total coliforms, fecal coliforms, and presumptive *Clostridium perfringens* has been reported (Frentzel et al., 2018; Garcia et al., 2001; Pafumi, 1986; Sospedra et al., 2010). Oregano is used in a variety of Mexican dishes (Rivero-Cruz et al., 2011) without any treatment for the reduction or elimination of pathogenic microorganisms; thus, oregano could act as a vehicle for pathogen transfer to foods.

Escherichia coli is one of the most important and widely studied etiologic agents of diarrhea worldwide (Jesser & Levy,

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2020). The diarrheagenic strains of *E. coli* are categorized into six pathotypes, collectively known as diarrheagenic *E. coli* pathotypes (DEP). DEP include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), enteroinvasive *E. coli* (EIEC), and Shiga toxin-producing *E. coli* (STEC, also referred to as enterohemorrhagic *E. coli* (EHEC)) (Gomes et al., 2016). Several DEP have been involved in outbreaks of diseases where herbs have been identified as the vehicle. The DEP involved in these outbreaks included STEC, which was responsible for an outbreak in Portugal where fresh parsley was identified as the vehicle. This outbreak resulted in 50 illnesses and three hospitalizations (EFSA BIOHAZ Panel et al., 2020). Also, ETEC was identified in an outbreak due to the consumption of fresh basil in Denmark that affected ca. 200 individuals (Yang et al., 2017). Due to the scarce information on the occurrence of DEP in herbs, it is necessary to carry out studies on other herbs, such as oregano.

Nontuberculous mycobacteria (NTM) represent an important group of environmentally saprophytic and potentially pathogenic bacteria that can cause serious mycobacteriosis in humans and animals (Pavlik et al., 2022a). They are characterized by exceptional adaptability and durability. They are capable of colonization and survival, even under highly unfavorable conditions (Pavlik et al., 2022b). Most cases of human mycobacteriosis occur in individuals with pre-disposing conditions, although NTM illness has been noted in apparently healthy individuals (Falkinham, 2022). Behavioral risk factors include smoking, excess alcohol consumption, and exposure to dusty occupations. Genetic factors include cystic fibrosis, α-1-antitrypsin deficiency, and deficiencies in the production of immune-signaling proteins. Infection by HIV is also a leading risk factor for NTM diseases (Falkinham, 2021). The incidence of NTM diseases is increasing worldwide (Kendall & Winthrop, 2013); for example, in the United States, the prevalence of NTM diseases has been rising from 8.2 per 100,000 persons in 1994 to 16 per 100,000 persons in 2014 (Donohue, 2018). Moreover, recently, Wang et al. (2022) reported that patients with NTM lung disease in Taiwan have increased mortality. While it has long been known that aquatic environments are a source of NTM that can infect people and animals, there is still insufficient knowledge of the ecology and sources of human exposure to many NTM species (Pavlik et al., 2022b). NTM have been isolated from various kinds of food, and many studies support the hypothesis that food, especially raw or partially cooked products, plays a role as a source of NTM for humans (Yoder et al., 1999; Argueta et al., 2000). Of note, the *M. avium* complex route of infection can occur through the respiratory or gastrointestinal tracts. In addition, disease dissemination can occur through the consumption of spring water, raw seafood, or hard cheese (Marochi-Telles et al., 2020). Some of the mycobacterial DNA fingerprints have been shown to be identical between patients and food isolates (Yoder et al., 1999). Due to the paucity of data on the microbial contamination and presence of specific pathogenic bacteria in oregano in Mexico, we conducted the current study with the following aims: to assess the microbiological quality and prevalence of *E. coli* in oregano samples purchased from different popular markets in Mexico City, to determine the occurrence in oregano of all six diarrheagenic *E. coli* pathotypes (DEP), and to evaluate the occurrence of NTM in this food item.

2 MATERIALS AND METHODS

2.1 Area of study and sample collection

The selected area of study was Mexico City, a large urban area of 9 million registered residents, whose population swells to nearly 25 million during working hours. Mexico City is divided into 16 boroughs, colloquially known as alcaldias in Spanish. From August 2021 to August 2022, a total of 85 oregano samples were purchased from popular markets in Mexico City (Figure 1): 5–6 samples from each borough. An amount of 100 g of oregano were collected from opened retail bags and placed into sterile sampling bags, which were placed in a rack for transportation to the laboratory.

2.2 Microbiological analysis

Notably, 10-g-weighed portions of samples were transferred to 90 mL of lactose broth in order to achieve a final 1:10 dilution (10^{-1}). Samples were homogenized for 2 min in a stomacher (tissue disrupter) and serially diluted (10^{-1} to 10^{-5}); these dilutions were then used for quantification (CFU/mL) and estimation (MPN/g) of microorganisms. Each sample was tested for the presence of aerobic-mesophilic bacteria (AMB), total coliforms (TC), fecal coliforms (FC), and *E. coli* following the methods approved by the Bacteriological Analytical Manual of the U.S. Food and Drug Administration (2021). The methods used for the isolation and identification of the microorganisms found in oregano samples are shown in Table 1. All confirmed *E. coli* strains were streaked onto tryptic soy agar slants, incubated at 37°C for 24 h, and maintained at 3–5°C until they were used for polymerase chain reaction (PCR).

2.3 Multiplex PCRs for the identification of DEP loci

E. coli strains isolated from the oregano samples were analyzed by two multiplex PCRs for the identification of DEP loci. The first multiplex PCR identifies the master regulon (*agg*R) of EAEC (Cerna et al., 2003), as well as the Afa adhesion usher (*afa*C) for DAEC (Patzi-Vargas et al., 2013). The reference strains for this multiplex PCR were EAEC 042 and DAEC C18451-A. The second multiplex identifies the following loci: Shiga toxin 1 and 2 (*stx*1, *stx*2) and intimin (*eae*A) for STEC, heat-stable and heat-labile enterotoxins (*st, lt*) for ETEC, intimin (*eae*A) and bundle-forming pilus (*bfp*A) for EPEC, and invasion-associated loci (*ial*) for EIEC (Lopez-Saucedo et al., 2003). The reference strains for the multiplex PCR were ETEC H10407, EPEC E2348-69, EHEC EDL933, and EIEC E11. PCR products were visualized by ethidium bromide staining after electrophoresis in a 2.5% agarose gel in Tris-acetate-EDTA buffer.

2.4 Isolation and identification of mycobacteria

A volume of 40 mL of supernatant from a 10^{-1} dilution was placed in 50-mL sterile conical centrifugation tubes (Falcon type) and then centrifuged (3,000 × *g* at room temperature for 30 min). Supernatants were discarded, and pellets were resuspended in 5 mL of 1% cetylpyridinium chloride solution, incubated at room temperature for 30 min, and neutralized

with 35 mL of PBS (pH 7.0). Samples were centrifuged as above, and pellets were resuspended in 5 mL of Dubos medium (Difco, Becton Dickinson, Sparks, MD) with albumin-dextrose-catalase (ADC; Becton Dickinson, Mexico); 100 µL of this suspension was inoculated onto Middlebrook 7H10 agar (Difco, Becton Dickinson) supplemented with ADC, cycloheximide (500 µg/mL), and the PANTA cocktail (Becton Dickinson) (40 U/mL polymyxin B, 4 µg/mL amphotericin B, 16 µg/mL nalidixic acid, 4 µg/mL trimethoprim, and 4 µg/mL azlocillin). Plates were incubated at 35°C and examined daily for the first 8 days and once a week thereafter for 2 months. Once bacterial growth had been observed on Middlebrook 7H10 agar, the identification of acid-fast bacilli was carried out by Ziehl-Neelsen staining. Acid-fast bacilli were subcultured on Middlebrook 7H10 agar and labeled by sampling location and with a consecutive number.

Figure 1. Some oregano samples purchased in markets in Mexico City.

Table 1. Methods used for isolation and identification of microorganisms in oregano samples.

Microorganisms	Presumptive test or primary enrichment	Confirmatory test or secondary enrichment	Selective medium for isolation	Identification test	Results reported as
Aerobic mesophilic bacteria	NR	Plate count agar (Bioxon™, BD, State of Mexico, Mexico) for 48 h at 35°C	NR	NR	CFU/g
Total coliforms	Lactose broth (Difco™, BD, Sparks, MD, USA) for 48 h at 35°C	Brilliant green lactose bile broth (Difco™, BD, Sparks, MD, USA) for 48 h at 35°C	NR	Gas production	MPN/g
Fecal coliforms	Lactose broth (Difco™, BD, Sparks, MD, USA) for 48 h at 35°C	*Escherichia coli* broth (Difco™, BD, Sparks, MD, USA) for 48 h at 44.5°C	NR	Gas production	MPN/g
Escherichia coli	Lactose broth (Difco™, BD, Sparks, MD, USA) for 48 h at 35°C	*Escherichia coli* broth with MUG (4-methylumbelliferyl-β-D-glucuronide) (Difco™, BD, Sparks, MD, USA) for 48 h at 44.5°C	Eosin methylene blue agar (Bioxon™, BD, State of Mexico, Mexico) for 24 h at 35°C	Indole production, methyl red test, Voges-Proskauer test, and citrate utilization (Bioxon™, BD, State of Mexico, Mexico) for 24 h at 35°C	MPN/g

NR: not required; CFU/g: colony-forming units per gram; MPN/g: most probable number per gram.

Strains belonging to the *Mycobacteriaceae* family and to the *Mycobacterium tuberculosis* complex were identified by two PCR assays (Cobos-Marin et al., 2003). The NTM species were identified by three methods:

- PCR restriction enzyme pattern analysis of the 65-kDa heat shock protein gene (*hsp*65), as described by Telenti et al. (1993);
- sequencing of the hypervariable region 2 (V2) of the 16S rRNA gene (Kirschner et al., 1993);
- sequencing of the *rpo*B gene (Adekambi et al., 2003).

Nucleotide sequences were compared to known sequences in the GenBank database by using the Blastn algorithm. Species identifications were based on the 100% similarity cutoff value for the 16S rRNA gene and ≥97% for the *rpo*B gene.

2.5 Statistical analysis

Descriptive statistics were used to summarize the data in the form of frequencies and percentages. The Spearman correlation coefficient (rho) value was calculated to quantify the relationship between the nominal (presence of NTM) and quantitative variables (concentration of AMB, TC, FC, and *E. coli*). Differences were considered significant at $p<0.05$. All statistical analyses were run with the statistical program Stata, version 14.0.

3 RESULTS

3.1 Microbiological quality and occurrence of DEP in oregano samples

All 85 samples analyzed were positive for AMB (Table 2). Concentrations of AMB ranged from 1.14 to 6.5 log CFU/g. A total of 67 (78.8%), 41 (48.2%), and 9 (10.6%) oregano samples were positive for TC, FC, and *E. coli*, respectively. TC, FC, and *E. coli* presented limits ranging from <3 to >1100 MPN/g (Table 2). A total of 46 *E. coli* strains were isolated from nine *E. coli*-positive samples, and all were genotyped for the presence of nine characteristic DEP loci; one sample was contaminated with STEC strains positive for the Shiga toxin 2 (*stx*2) locus at a concentration of 3 MPN/g.

Table 2. Aerobic mesophilic bacteria (AMB), total coliforms (TC), fecal coliforms (FC), *Escherichia coli*, diarrheagenic *E. coli* pathotypes (DEP) concentrations, and number of positive samples in oregano samples*.

Microorganisms	Minimum	Median	Maximum	Number of positive samples (%)
AMB	1.14	4.17	6.5	85 (100)
TC	<3	43	>1,100	67 (78.8)
FC	<3	<3	>1,100	41 (48.2)
E. coli	<3	<3	>1,100	9 (10.6)
DEP	<3	<3	3	1 (1.1)

*n = 85. Minimum, median, and maximum values are in $\log_{10}$ CFU per gram for AMB and most probable number (MPN) per gram for TC, FC, *E. coli* and DEP.

3.2 Mycobacteria isolation and identification

Of the 85 oregano samples analyzed, 15 (17.6%) yielded mycobacterial strains, one strain per sample. *Mycolicibacterium fortuitum* was the most frequently isolated organism (six strains). We also recovered two strains of *Mycolicibacterium smegmatis*, and one strain of each of the following species: *Mycolicibacterium conceptionense*, *Mycolicibacterium porcinum*, *Mycolicibacterium parafortuitum*, *Mycolicibacterium flavescens*, and *Mycolicibacterium goodii*. Moreover, two mycobacterial species could not be identified by the molecular methods used (Table 3). No correlation between the presence of NTM and the presence of AMB (rho=0.179, p=0.100), TC (rho=0.160, p=0.143), FC (rho=0.1656, p=0.129), and *E. coli* (rho=0.1703, p=0.1193) was found.

4 DISCUSSION

The environmental reservoirs and behavioral risk factors associated with NTM disease are not well understood. More epidemiological research is necessary to identify specific environmental reservoirs for the various NTM species, which may help prevent transmission to susceptible individuals (Koh, 2013). Therefore, the present study focused on assessing the microbiological quality and presence of DEP and NTM in oregano samples purchased from different markets in Mexico City.

In this study, AMB was detected in 100% of the oregano samples analyzed, with concentrations between 1.14 and 6.5 log CFU/g. These results for AMB are similar to those found by Dinh Thanh et al. (2018), Garbowska et al. (2015), Pafumi (1986), Sospedra et al. (2010), and Witkowska et al. (2011), in oregano samples from Germany, Poland, Australia, Spain, and Ireland, respectively. Unfortunately, in Mexico, there is no national guideline that establishes the maximum permissible limits for microorganisms in dried spices and herbs. The high AMB levels found in this study may have been due to the exposure of most oregano samples to the environment and storage at room temperature, which may have led to the proliferation of AMB, resulting in increased bacterial counts. AMB is an indicator group related to overall food quality and a lack of hygiene during the production process; therefore, it is necessary to implement sanitation practices for oregano.

A total of 67 (78.8%), 41 (48.2%), and 9 (10.6%) oregano samples were positive for TC, FC, and *E. coli*, respectively, with limits ranging from <3 to >1,100 MPN/g. Coliform bacteria are commonly used as an indicator of the sanitary quality of foods or to check for potential contamination of pathogenic microorganisms. Additionally, the presence of *E. coli* in food indicates the possibility that fecal contamination has occurred and that other microorganisms of fecal origin, including pathogens, may be present. TC and *E. coli* counts were found in this study to be higher than those reported by Pafumi (1986) for oregano samples from Australia, who reported the presence of coliforms in four of seven (57%) samples analyzed, with concentrations between 3 and 100 MPN/g and the absence of *E. coli*.

A total of 46 *E. coli* strains were isolated from nine *E. coli*-positive samples, and all were genotyped for the presence of nine characteristic DEP loci; one sample was contaminated with STEC strains positive for the Shiga toxin 2 (*stx*2) locus

Table 3. Species of nontuberculous mycobacteria identified in oregano samples.

Strain	Origin	16S rRNA	*hsp65*	*rpo*B	Species identified
AVM1	Xochimilco	*M. fortuitum*	*M. fortuitum*	*M. fortuitum*	*M. fortuitum*
AVM8	Milpa Alta	*M. conceptionense*	*M. conceptionense*	*M. conceptionense*	*M. conceptionense*
AVM9	Milpa Alta	*M. porcinum*	*M. porcinum*	*M. porcinum*	*M. porcinum*
AVM18	Cuajimalpa	*M. smegmatis*	*M. smegmatis*	*M. smegmatis*	*M. smegmatis*
AVM23	Tlahuac	*M. parafortuitum*	*M. parafortuitum*	*M. parafortuitum*	*M. parafortuitum*
AVM46	Tlalpan	*M. fortuitum*	*M. fortuitum*	*M. fortuitum*	*M. fortuitum*
AVM48	Tlalpan	*M. fortuitum*	*M. fortuitum*	*M. fortuitum*	*M. fortuitum*
AVM49	Tlalpan	*M. fortuitum*	*M. fortuitum*	*M. fortuitum*	*M. fortuitum*
AVM50	Tlalpan	*M. fortuitum*	*M. fortuitum*	*M. fortuitum*	*M. fortuitum*
AVM54	Benito Juarez	No close reference	*M. nonchromogenicum*	No close reference	*M.* sp.
AVM55	Benito Juarez	No close reference	*M. nonchromogenicum*	No close reference	*M.* sp.
AVM59	Miguel Hidalgo	*M. fortuitum*	*M. fortuitum*	*M. fortuitum*	*M. fortuitum*
AVM79	Iztacalco	*M. flavescens*	*M. flavescens*	*M. flavescens*	*M. flavescens*
AVM81	Iztapalapa	*M. goodii*	*M. goodii*	*M. goodii*	*M. goodii*
AVM82	Iztapalapa	*M. smegmatis*	*M. smegmatis*	*M. smegmatis*	*M. smegmatis*

at a concentration of 3 MPN/g. To the best of our knowledge, this is the first study to identify the presence of STEC strains in oregano. STEC are estimated to cause more than 1.2 million illnesses and 128 deaths globally each year (FAO & WHO, 2022). STEC is the main causative pathogen of diarrhea-associated hemolytic uremic syndrome (HUS). STEC-associated HUS is a clinical syndrome involving hemolytic anemia (with fragmented red blood cells), low levels of platelets in the blood (thrombocytopenia), and acute kidney injury (AKI). It is the major infectious cause of AKI in children. In severe cases, neurological complications and even death may occur (Liu et al., 2022). Therefore, measures to reduce or eliminate STEC strains from oregano samples should be implemented. Some studies have shown that radio frequency (RF), which is an electromagnetic wave with frequencies of 1–300 MHz, is useful for reducing *E. coli* O157:H7 in food (Kim & Song, 2023). Tong et al. (2022) used a RF system to inactivate *E. coli* O157:H7 and *Salmonella Typhimurium* in black pepper kernels. They reported a reduction of *E. coli* O157:H7 and *Salmonella Typhimurium* in more than 6 log CFU/g after RF heating for 7.0 and 8 min, respectively, with no significant influence on the quality of black pepper kernels. Thus, the RF may be used to reduce STEC in oregano.

The incidence of NTM infections has increased to similar or higher levels than that of *Mycobacterium tuberculosis* complex infections in England, the United States, and several European countries in the past decade (Jarchow-MacDonald et al., 2023). In this study, *M. fortuitum*, *M. smegmatis*, *M. conceptionense*, *M. porcinum*, *M. parafortuitum*, *M. flavescens*, and *M. goodii* were identified in the oregano samples. To the best of our knowledge, this is the first report of isolation of NTM from oregano. *M. fortuitum*, *M. conceptionense*, and *M. porcinum* belong to the *Mycobacterium fortuitum* group, which are able to cause clinical mycobacteriosis in fish and other animals, including humans (Pavlik et al., 2021). In particular, *M. fortuitum*, the mycobacterial species most isolated in this study, is the main NTM associated with skin and soft-tissue infections worldwide (Kumar et al., 2021). Additionally, *M. fortuitum* has been isolated from patients with NTM illness (Escobar-Escamilla et al., 2014; Lopez-Luis et al., 2020) and from food items (Cerna-Cortes et al., 2015; Cerna-Cortes et al., 2016; Cerna-Cortes et al., 2019) in Mexico City. Future studies of DNA fingerprinting of NTM should be performed to confirm that NTM isolated from oregano are the same as those isolated from patients. In terms of microbiological safety and hygiene, the presence of mycobacteria in foods represents a potential biological hazard that should be prevented.

5 CONCLUSION

Our results show that the oregano samples analyzed had unsatisfactory microbiological quality, and some harbored STEC and NTM strains associated with illness. Oregano could be considered a potential source of NTM infections in humans. Measures to diminish or eliminate the high levels of microorganisms and pathogenic bacteria in this food item are advisable. Good hygiene practices, good manufacturing practices, and hazard analysis and critical control points are crucial and must be applied throughout the chain of production. These measures must be combined with microbial inactivation treatments such as the use of RF to ensure the safety of oregano.

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