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Calidad microbiana de *Lactuca sativa* en el mercado 9 de octubre en la ciudad de Cuenca-Ecuador

Microbial quality of Lactuca sativa in the 9 de Octubre market in the city of Cuenca-Ecuador

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Palabras claves:
Mohos, Levaduras, Enteroparásitos, hortaliza, intoxicación alimentaria, Compact Dry YM.

Resumen

Introducción: Los hongos y levaduras son organismos de gran interés clínico, ya que originan sustancias tóxicas para la salud humana, las micotoxinas. Las enteroparasitosis son infecciones intestinales producidas por parásitos. La principal fuente de transmisión de estos microorganismos son los alimentos crudos. La lechuga es una importante hortaliza de cultivo a nivel mundial, ya que es parte de una dieta saludable. **Objetivo:** Determinar la presencia de mohos, levaduras y parásitos en *Lactuca sativa* (lechuga) expendidos en el mercado 9 de octubre, ubicado en el centro histórico de Cuenca, provincia del Azuay. **Metodología:** Estudio descriptivo transversal, con información en el análisis realizado de las hortalizas expendidas en el mercado 9 de octubre. Para la identificación y cuantificación de hongos se empleó placas Compact Dry YM y mediante microscopía se determinó la existencia de parásitos en las 30 muestras de lechuga recolectadas. **Resultados:** En base al estudio realizado, se evidencio el crecimiento de mohos y levaduras en las 30 muestras analizadas en las placas Compact Dry YM. Se demostró que son aptas para el consumo humano puesto que se encuentran dentro de los límites permisibles de la normativa del gobierno vasco Por otro lado, con relación al análisis parasitario, alrededor del 60% de las muestras presentaron quistes de *Entamoeba coli*. **Conclusión:** Se determinó la contaminación de levaduras, mohos y parásitos en la lechuga demostrando que en este establecimiento público no está teniendo una correcta práctica higiénica. Está contaminación puede darse en cualquier etapa, durante el cultivo, transporte y/o almacenamiento y manipulación de por parte de los vendedores que expenden esta hortaliza en dicho mercado. **Área de estudio general**: Bioquímica y Farmacia. **Área de estudio específica:** Microbiología de alimentos. **Tipo de estudio:** Artículo original.

Keywords:
Molds, Yeasts, Enteroparasites, vegetables, food poisoning.

Abstract

Introduction: Fungi and yeasts are organisms of great clinical interest since they originate toxic substances for human health (mycotoxins). Enteroparasites are intestinal infections caused by parasites. The primary source of transmission of these

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microorganisms is raw food. Lettuce is a crucial cultivated vegetable worldwide, as it is part of a healthy diet.**Objective:**To determine the presence of molds, yeasts, and parasites in Lactuca sativa (lettuce) sold in the '9 de Octubre' market, located in the historic center of Cuenca, province of Azuay.**Methodology:**A cross-sectional descriptive study was conducted through the analysis of vegetables sold in the '9 de Octubre' market. Compact Dry YM plates and microscopy were used to identify and quantify fungi in the 30 lettuce samples collected to determine the existence of parasites.**Results:**Based on the study, the growth of molds and yeasts was evidenced in the 30 samples analyzed. It was demonstrated that they are suitable for human consumption since they are within the permissible limits of the Basque Government regulations. On the other hand, in the parasitic analysis, about 60% of the samples showed Entamoeba coli cysts.**Conclusion:**The contamination of yeasts, molds, and parasites in lettuce was determined, demonstrating that this public establishment does not have a correct hygienic practice. This contamination can occur at any stage, during cultivation, transportation, and/or storage and handling by vendors.**General study area**: Biochemistry and Pharmacy.**Specific area of study**: Food microbiology.**Study type:**Original article.

Introduction

Fungi are widely distributed in nature. They are contaminating agents capable of degrading a wide variety of organic substrates, such as fruits, vegetables and greens. We can differentiate two types of fungal organisms with their own characteristics that differentiate them, molds and yeasts (1).

Molds or filamentous fungi, as their name indicates, are multicellular organisms made up of branched microscopic filaments with defined cell walls. They are heterotrophs, meaning they feed on other organisms. One characteristic of these living beings to highlight is the production of spores, a mechanism that allows them to survive as resistant cells until they achieve better growth conditions. On the other hand, yeasts are unicellular fungi, with a spherical appearance. They are identified by the formation of hyphae and

pseudohyphae. Their growth is observed 24 to 36 hours after incubation, a distinctive feature that differentiates them from molds since the latter grow more slowly (1, 2).

The danger of these microorganisms is related to the production of mycotoxins, substances that cause acute poisoning in animals and have mutagenic, neurotoxic, immunosuppressive and carcinogenic effects on human health. The main genera of molds that produce mycotoxins are: Aspergillus spp., Fusarium spp. and Penicillium spp; which produce substances such as: aflatoxins, ochratoxins A, fumonisins, T-2 toxin and Zearalenone, most frequently found in contaminated foods of plant origin, cereals, nuts, vegetables and fruits; and of animal origin, in the case of animals that have consumed contaminated feed (3, 4).

Parasites are commensal individuals lodged inside another living being called host. Their relevance refers to intestinal infections caused by the ingestion of protozoan cysts and helminth eggs, called enteroparasitosis (5).

Protozoa are parasites with a wide variety of shapes and sizes. Their main characteristic is the formation of cysts, which are responsible for their dissemination since they have the ability to survive long periods of time in the environment without losing their effectiveness in causing diseases through infection of animals or humans, or in turn through vehicles such as contaminated food. Helminths are worm-shaped parasites that live inside their host (5, 6).

The distinctive clinical manifestations of parasitosis are malnutrition, diarrhea, malabsorption, abdominal pain and nausea. Montenegro Concha et al, in their research establish a relationship between the parasite that causes the disease and the symptoms it presents, thus indicating that infections produced by Entamoeba hystolitica experience severe diarrheal symptoms (7).

The main source of transmission of these organisms is raw food, which serves as a vehicle for microorganisms to enter the human body. The health and hygiene of workers, poor sanitary practices carried out; and the use of contaminated water are relevant factors since they facilitate food contamination. Foodborne diseases (ETA) are diseases caused by the ingestion of food contaminated by microorganisms or chemical substances. They are of great relevance within public health since they cause high mortality rates globally. In 2021, in Ecuador about 3,152 cases of ETA were determined, the province of El Oro was the most affected city with 1,257 cases, followed by Guayas with 436 cases, Loja (124 cases) and Azuay (43 cases) (8).

In Ecuador and worldwide, water is very important in agriculture. Despite being a fundamental resource, there is a deficiency in its quality since it is not subjected to any technological process. Irrigation water, commonly known, is collected from the river and

is used to irrigate foods of plant origin. Being a type of untreated water, it can present bad odors, strange flavors, turbidity, and research has even determined the presence of toxic products and pathogenic microorganisms, the latter coming from contamination through human and animal excrement. The usual parasitic form of transmission is eggs or cysts, capable of causing diseases of clinical interest. Entamoeba coli is the parasite most frequently observed in irrigation water (9).

Lettuce (Lactuca sativa) is an important vegetable crop grown worldwide, as it is part of a healthy diet. For the reasons mentioned above, it is important to determine the presence of molds, yeasts and parasites in vegetables commonly sold in these establishments and to know the risks that their ingestion entails for humans. This research was carried out in the city of Cuenca, province of Azuay, in the 9 de Octubre market, a public market that is highly visited due to its wide variety of products and its low economic cost, and lettuce was considered one of the most sold vegetables since it is used in different types of dishes as a fresh vegetable, in addition to its nutritional value, rich in vitamins and proteins.

Methodology

This research was cross-sectional, of a descriptive observational type. The study was developed from samples obtained from the 9 de Octubre market in the city of Cuenca - Azuay, Ecuador. A non-probabilistic sampling was carried out for convenience. In this context, 30 lettuce samples were collected,obtainedrandomly from the sales stands belonging to the aforementioned public establishment.

Methods and techniques

Compact Dry YM plates are chromogenic media that differentiate different microorganisms by means of the substrates they contain: X-Phos, have a blue color characteristic for the growth of yeasts, compared to molds that take a three-dimensional brown form. Both fungal organisms have an incubation time of 3 to 7 days at a temperature of 25 to 30°C (10).

Lugol's staining is a technique used to identify intestinal parasites. Its basis is based on the formation of complexes with carbohydrates from the cysts and eggs of these living beings, observing a dark brown coloration of the main components of the parasites, thus facilitating their observation under a microscope. This method is mainly used for the determination of Entamoeba histolytica cysts (11).

Sampling

The lettuce samples were obtained directly, under aseptic conditions. As it is a food formed in layers, it is necessary to take samples of each of its leaves in equal proportions.

For microbiological analysis, the food was transported to the Microbiology laboratories of the Biochemistry and Pharmacy program at the Catholic University of Cuenca.

Sample preparation

First, 10 grams of the food (sample) was weighed. on a calibrated scale and placed in a stomacher with 90 mL of sterile peptone water, prepared previously. The food was then liquefied for two minutes and then left to rest for 15 minutes so that the large particles would settle. To prepare the following dilutions, the surface layer formed was used.

- **Initial dilution or 1/10**: Using a sterile pipette, 1 mL of the initial suspension was transferred to a tube containing 9 mL of previously placed sterilized peptone water and the mixture was homogenized. In this way, a 10% dilution was obtained. It should be noted that each successive dilution will decrease its concentration by 10 times.
- **Dilution 1/100**: repeating the same procedure, 1 mL of the 1/10 dilution was transferred to another tube containing 9 mL of peptone water and homogenized.
- **Dilution 1/1000**: Likewise, 1 mL of the 1/100 suspension was transferred to another tube containing 9 mL of sterile diluent and homogenized.

For the parasite analysis, 5 mL of each sample was taken from the stock dilution prepared at the beginning and placed in a test tube and left to rest for 24 h for better visualization under the microscope. After the resting time, centrifugation was carried out for 10 minutes at 3000 rpm, then the supernatant was discarded by decantation and the analysis sample was prepared. For this, a drop of the final sample was placed together with a drop of Lugol's reagent on a coverslip and examined with the 10X and 40X objectives (12).

Technical standard of the Basque Government

Due to the lack of national regulations that delimit the optimal permissible range of microbial load in relation to molds and yeasts for human consumption, the technical standard of the Basque Government, Portugal, was taken as a reference. The Department of Health of this government offers a control regulation for food, showing the microbiological limits and physical-chemical quality parameters of each of them. Through this document, compliance with the established requirements is regulated, in this case for the analysis of fresh vegetables and greens suitable for human consumption (13). See table 1.

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Table 1.*Microbiological requirements for vegetables and greens taken from the Basque Government Regulations.*

Microorganism	Permissible limit
Molds and yeasts	Molds/Yeasts: 10 x 104 CFU/g Molds: non-toxigenic strains

Regarding parasites, these are determined by their presence or absence in the food of interest and therefore do not present permissible limits.

Results

Based on the study, the growth of molds and yeasts was evident in the 30 samples analyzed on the Compact Dry YM plates. See figure 1.

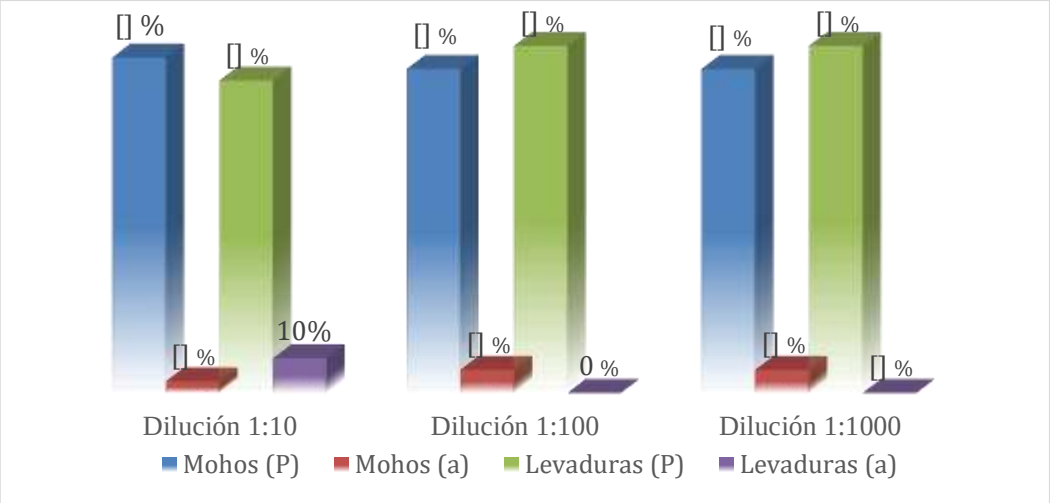

Figure 1. Total representation of the samples according to the growth of molds and yeasts from the 9 de Octubre Market in the city of Cuenca. The initials (P) refer to the presence of such microorganisms in the sample and the letter (a) to the absence.

Based on this, we identified the presence of 100% of yeasts in the 1:100 and 1:1000 dilutions and at least 90% of molds in the three types of dilutions and yeasts in the 1:10 dilution. It should be noted that in most of the plates uncountable values were observed, that is, there was excessive growth of colonies that prevented their quantification.

Identification and quantification of molds and yeasts

For the identification and quantification of these microorganisms, the instructions declared by the manufacturer were followed. To perform the count from the back of the plate, a number of 15-150 colony forming units (CFU) had to be counted, otherwise the dilution was discarded. Most of the yeasts were observed with a blue pigmentation and in the case of molds, a brown color. See figure 2.

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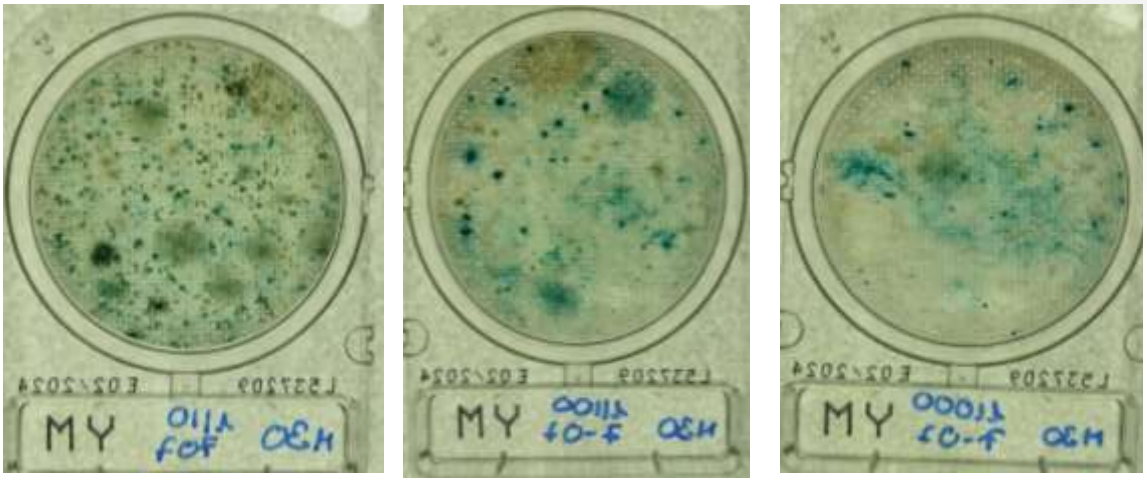

Figure 2. Growth of moulds and yeasts on Compact Dry YM plates. The first image represents the 1/10 dilution, the second the 1/100 dilution and the third the 1/1000 dilution. The brown colonies symbolise mould growth and the blue colonies refer to yeasts.

Based on the above, it was possible to calculate 11 samples, given that the rest showed excessive growth and were determined to be uncountable. As a result, it was found that the samples were within the permissible limits established by the Basque government regulations, which state that a food must not exceed 10 x 104 CFU of molds and yeasts. It is important to indicate that this regulation was taken as a reference since at a national level there is no manual that establishes the suitable limits for these fungal organisms. See table 2.

Table 2. Calculations applied to the 11 samples based on the formula and its relationship withhelimit permissible under the Basque Government regulations.

Sample number	Dilution used	Application of the formula (UFC/g)	Set limit, value within range 10 x 104 (Acceptable / Not Acceptable)
1	1/1000	3.6 x 104	Yeah
4	1/1000	5 x 104	Yeah
5	1/1000	2.9 x 104	Yeah
19	1/1000	5.1 x 104	Yeah
20	1/10 1/1000	3.9 x 104	Yeah
21	1/10 1/1000	1.1 x104	Yeah
22	1/1000	7.6 x 104	Yeah
23	1/100	3 x104	Yeah
24	1/1000	5.5 x 104	Yeah
25	1/100	8.2 x 101	Yeah
26	1/1000 1/100	6,1 x 102	Yeah

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On the other hand, in relation to the determination of enteroparasites in lettuce, in around 60% of the samples, the existence of Entamoeba coli cysts was observed by microscopy. See figure 3.

Photo A: **Photo B:**

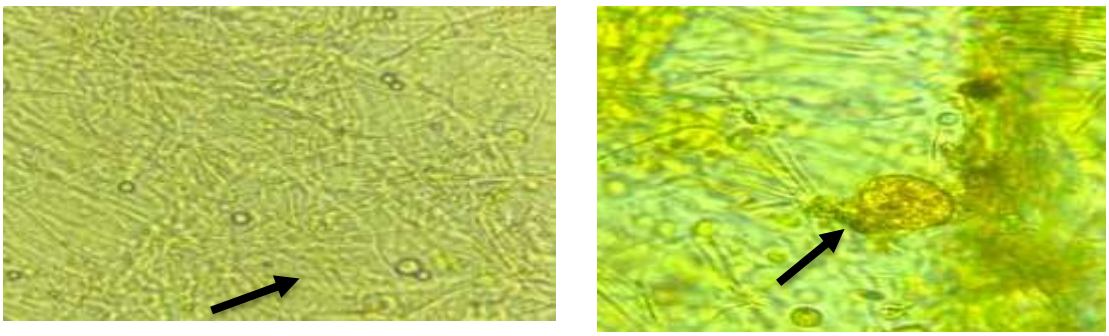

Figure 3. Microscope photographs, yeast (A), E. coli cyst (B).

Discussion

The present study carried out in the 9 de Octubre market, Cuenca, had the purpose of demonstrating the presence of molds, yeasts and enteroparasites in the lettuces sold in this establishment. The positive existence of 100% of fungal microorganisms was obtained, and around 60% of*Entamoeba coli*,suggesting high microbial contamination, indicative of aindirect fecal-oral transmission,on the partof andthis market (14).

A study carried out by Gabre RM in Saudi Arabia shows that fresh vegetables used for salads are the foods most contaminated by parasites. Thus, the presence of 184 parasites (46%) in 400 vegetables was determined, among which 23 cysts (12.50%) of Entamoeba coli were observed (14). In Brazil, a research where 32 samples of lettuce from agroecological and traditional fairs were collected, the existence of 10 species of parasites was observed in 23 samples (71.8%), the main one being Entamoeba coli with 53% and lower percentages Iodamoeba butschlii, Endolimax nana and Entamoeba hartmanni. E. histolyrica/ E. dispar (15). Similarly, in the same country,Machado N et. collected224 parasite structures in 38 positive samples among 40 samples analyzed, resulting in a contamination rate of 95% by Entamoeba spp (16).

Vegetables have a microbial load of parasites. Entoameba spp is the most common parasitic organism as it could be observed, mainly due to unhygienic practices.food preparationandto inadequate storage conditions. It is a pathogenic parasite responsibleof most cases of human amoebiasis and remains one of the three main causes of parasitic mortality worldwide associated with the consumption of contaminated vegetables and fruits (17,18).

In Ecuador, parasitosis affects 80% of the population in rural areas and 40% in urban-marginal areas. The problem of this situation is related to water contamination by excrement and lack of sanitary conditions and sociocultural customs. In the province of Manabí, 62 samples of lettuce were sold from the municipal market of Portoviejo, observing the presence of parasites in lettuce from 51 samples, which corresponds toan 82.3%; while 11 lettuces had no parasitic presence equivalent to 17.7%, within which it was observed*Entamoeba coli*from 6.45%(19). Another study, carried out in the City of Cuenca, in four public markets with a total of 144 lettuce samples, resulted in:the presence of parasites as the main protozoa observed are Entamoeba spp cysts 19.03% (20).

On the other hand, regarding the microbial quality of molds and yeasts, Sirsat SA et al. in a study carried out in Houston - USA with the purpose of comparing the microbial quality and safety of lettuce in different vegetable sales stands from different socioeconomic areas classified as high and low by applying Petrifilm 3M plates, examined a total of 90 lettuce samples where a total of 100% were positive for low class establishments such as public markets and 53% for high class establishments (21). Similarly, in Portugal-Spain, a research carried out by Ferreira C et al. showed the existence of 100% positive for molds and yeasts in 20 lettuce samples analyzed (22).

At a national level, there are no updated studies that analyze yeasts and molds in lettuce, which is why this topic was chosen in order to obtain information about these microorganisms. As was observed internationally and in the present field research, there is a significant presence in lettuce, becoming a concern for the health of the population, in this case in the City of Cuenca.

Conclusion

- At the 9 de Octubre market, it was determined that lettuce (Lactuca sativa) samples had yeasts, molds and parasites, indicating that the vendors at the establishment were not applying proper hygiene practices. This contamination can occur at any stage during cultivation, transportation, etc. The determination of yeast and mold contamination was carried out using Compact Dry YM plates, and it was particularly worrying that the percentages of all dilutions 1:10, 1:100 and 1:1000 exceeded the acceptable concentration limits for government regulations.Basque. On the other hand, the existence of parasites in Lactuca sativa was confirmed by viewing a microscope using Lugol on a coverslip and examining it with 10X and 40X objectives, showing positive results for these microorganisms.
- To ensure a safe product, good hygiene practices must be applied by farmers, sellers and consumers to avoid contamination. Whenever possible, farmers should be encouraged to implement more effective control strategies for lettuce, considering factors such as fungal inhibitors, genetic selection, raw material

treatments, pesticide application, good harvesting, drying, distribution and storage practices to ensure consumer health.

Conflict of interest

There is no conflict of interest.

Authors' contribution statement

All authors contributed equally to the research.

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