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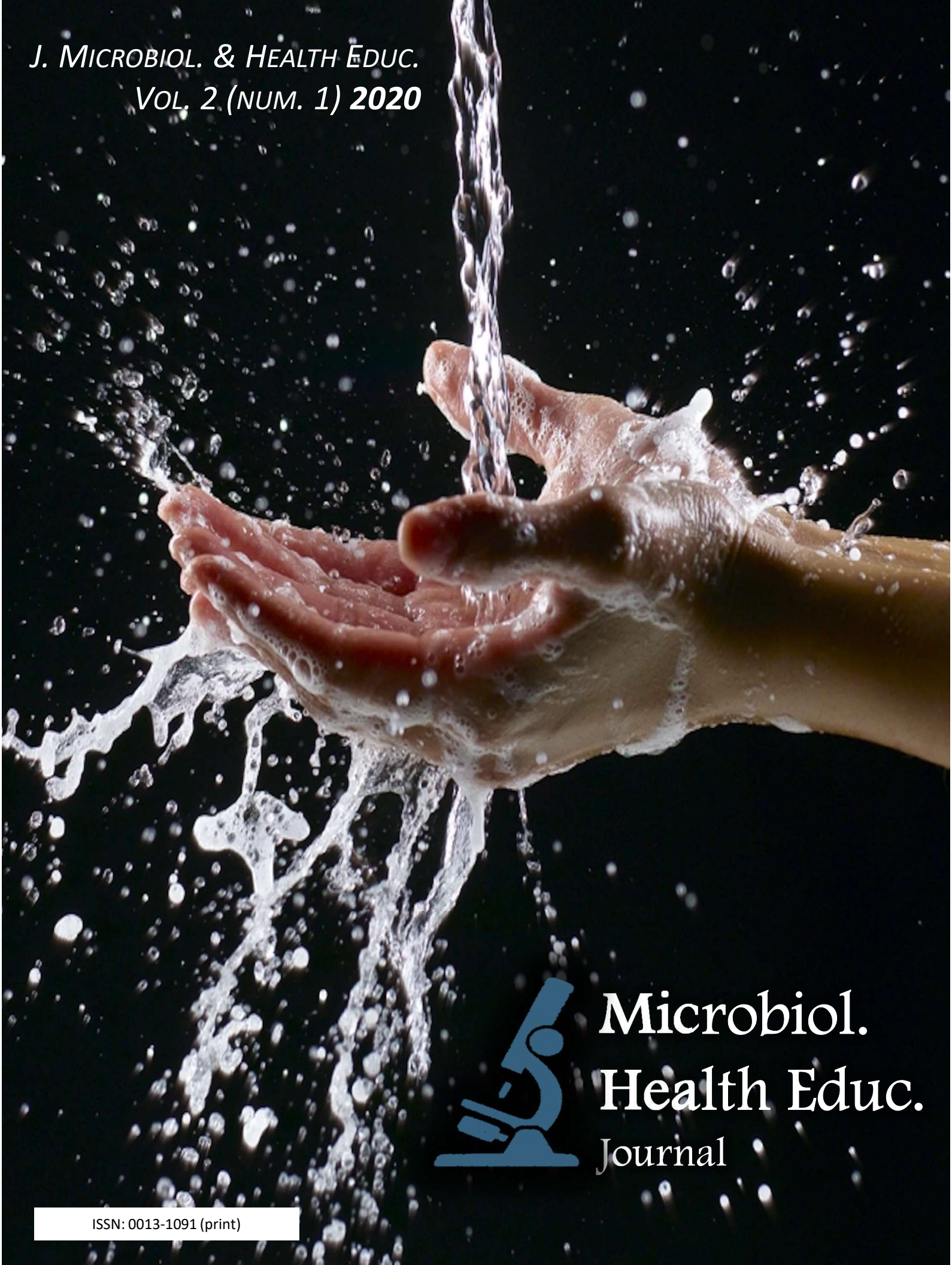

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ORIGINAL ARTICLE

Bacillus cereus in Samples of Pre-cooked and Cooked Rice for Sale to the Public in Colima State, Mexico

Bacillus cereus en muestras de arroz pre-cocido y cocido para su venta al público en Colima, México

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A B S T R A C T

Bacillus cereus is recognized as a pathogen that causes food poisoning. There have been outbreaks associated with the consumption of rice contaminated with this bacterium. It is one of the few pathogens with the ability to spoil food. Outbreaks of diarrheal and emetic syndromes caused by this organism have been widely reported in Mexico, Japan, the United States, the Netherlands, Thailand, Africa, South Korea, the United Kingdom, Belgium, and Finland. In Mexico, there are no data available to evaluate the epidemiological importance of *B. cereus* as a causative agent of enteric diseases, although its possible presence in parboiled and cooked rice could represent a health risk. Thus, the objective of this work was to identify the presence of *B. cereus* in parboiled and cooked rice for sale to the public in Colima, Mexico. The health quality, the quantity of *B. cereus* present and the identification of strains of the *hblA* and *ces* genes, which are involved in diarrheal and emetic syndrome, respectively, were determined. 155 samples of cooked and 32 precooked rice were collected from different establishments in the city of Colima (homes, fixed and street restaurants, inns and supermarkets). Sanitary quality was evaluated by observing the counts of aerobic mesophilic bacteria and total coliform bacteria, which were carried out following the current official methodology. The B. cereus count was performed on MYP agar (mannitol, yolk egg and polymyxin). Finally, the identification of diarrheal or emetic *B. cereus* strains was by amplification of the *hblA* and *ces* genes by PCR. In 87.5 % of the parboiled rice samples and in 23.2 % of the cooked rice samples, high counts of aerobic mesophilic bacteria were obtained (> 10^5 CFU / g), and in 71 % of the total samples analyzed it was demonstrated the presence of viable total coliform bacteria. In 28.1 % of the parboiled rice samples and in 89 % of the cooked rice samples, *B. cereus* was recovered. The *hblA* gene (encoding one of the three BL hemolysin subunits) was amplified in 86 % of *B. cereus* isolates, however, no strains carrying the *ces* gene (encoding cereulide) were identified. All samples of the cooked or parboiled rice samples containing vegetables (corn, potato, green bean, carrot and pea) showed the presence of *B. cereus*, so these ingredients may be related to the presence of the pathogen in cooked rice or precooked. Even in figures far below those considered as a health risk, if it is stored under conditions of temperature abuse, as is often the case in the sampled sites, contaminated rice can represent a risk to the health of the consumer.

Keywords: *Bacillus cereus*, cooked rice, parboiled rice, foodborne illness, food poisoning, diarrheal syndrome, emetic disease.

R E S U M E N

Bacillus cereus es reconocido como un agente patógeno causante de intoxicaciones alimentarias. Se han presentado brotes asociados al consumo de arroz contaminado con esta bacteria. Es uno de los pocos patógenos con capacidad de deteriorar los alimentos. Se han informado ampliamente brotes de síndromes diarreicos y eméticos causados por este organismo en México, Japón, Estados Unidos, Países Bajos, Tailandia, África, Corea del Sur, Reino Unido, Bélgica y Finlandia. En México, no existen datos disponibles para evaluar la importancia epidemiológica de *B. cereus* como agente causante de enfermedades entéricas, aunque su posible presencia en arroz precocido y cocido podría representar un riesgo para la salud. Así, el objetivo de este trabajo fue identificar la presencia de *B. cereus* en arroz precocido y cocido para venta al público en Colima, México. Se determinó la calidad sanitaria, la cantidad de *B. cereus* presentes y la identificación de cepas los genes *hblA* y *ces*, que están involucrados en el síndrome diarreico y emético, respectivamente. Se recolectaron de 155 muestras de arroz cocido y 32 precocido de diferentes establecimientos de la ciudad de Colima (hogares, restaurantes fijos y ambulantes, fondas y supermercados). La calidad sanitaria se evaluó observando los recuentos de bacterias mesofílicas aerobias y bacterias coliformes totales, que se realizaron siguiendo la metodología oficial vigente. El recuento de *B. cereus* se realizó en el agar MYP (manitol, yema de huevo y polimixina). Finalmente, la identificación de las cepas de *B. cereus* diarrogénicas o eméticas fue mediante amplificación de los genes *hblA* y *ces* por la técnica de reacción en cadena de la polimerasa. En el 87.5 % de las muestras de arroz precocido y en el 23.2 % de las muestras de arroz cocido se obtuvieron recuentos altos de bacterias mesofílicas aerobias (>10^5 UFC/g), y en el 71 % del total de las muestras analizadas se demostró la presencia de bacterias coliformes totales viables. En el 28.1 % de la muestras de arroz precocido y en el 89 % de las muestras de arroz cocido se recuperó *B. cereus*. El gen *hblA* (que codifica una de las tres subunidades de hemolisina BL) se amplificó en el 86 % de *B. cereus* aislados, sin embargo, no se identificaron cepas portadoras del gen *ces* (que codifica la cereulida). Todas las muestras de arroz cocido o precocido que contenían verduras (elote, papa, ejote, zanahoria y chícharo) mostraron presencia de *B. cereus,* por lo que estos ingredientes pueden estar relacionados con la presencia del patógeno en el arroz cocido o precocido. Aun en cifras muy por debajo de las consideradas como un riesgo para la salud, si se almacena en condiciones de abuso de temperatura, como suele ocurrir en los sitios muestreados el arroz contaminado puede representar un riesgo a la salud del consumidor.

Palabras clave: *Bacillus cereus*, arroz cocido, arroz precocido, enfermedad transmisible por alimentos, intoxicación alimentaria, síndrome diarreico, enfermedad emética.

INTRODUCTION

Bacillus cereus is one of the microorganisms involved; it can cause intestinal and extra-intestinal symptoms. Among the intestinal alterations, food poisoning is characterized by diarrhea (due to various enterotoxins, such as nonhemolytic enterotoxin, the hemolysin BL [HBL] complex, cytotoxin K, enterotoxin T, and enterotoxin FM (1, 2) and/or emetic (due to cereulide) syndromes (3, 2). The number of *B. cereus* cells capable of causing disease has been estimated to range between 10^4 and 10^8 cells per g of food (4). Outbreaks of diarrheal and emetic syndromes caused by this organism have been widely reported in Mexico (5), Japan (6), the United States (7), The Netherlands (6), Thailand (8), Africa (9), South Korea (10), the United Kingdom (11, 12), Belgium (13), and Finland (14). Outbreaks caused by *B. cereus* have usually been associated with rice or other grains and vegetables that contain starch in their composition (15). About 95% of emetic-type food poisoning is due to the consumption of rice, macaroni, cheese, and vanilla pudding (16, 17, 2), whereas outbreaks of the diarrheal type are most often related to spices, fruits, nuts, grains, dairy products, soups, and dry foods, although vegetables have also been involved (18, 19). The increase in the consumption of foods with a mild thermal treatment or that are minimally processed and refrigerated has favored the increase of spore-forming pathogenic bacteria, including some strains of psychrotrophic *B. cereus* (12, 20). Similarly, pasteurization of the final container eliminates the risks arising from vegetative pathogenic bacteria but is insufficient for the removal of spores, including those of *B. cereus* (13). In Mexico, there are no data available to assess the epidemiologic importance of *B. cereus* as the causative agent of enteric diseases, even though its possible presence in pre-cooked and cooked rice could represent a health risk.

Purpose of the study

The objective of this work was to determine the sanitary quality and the presence of *Bacillus cereus* strains that carry the *hblA* and *ces* gene, which are involved in diarrhea and emetic syndrome, respectively, in parboiled and cooked rice for sale to the public in Colima. Mexico.

MATERIALS AND METHODS

Sampling

155 samples of cooked rice and 32 precooked rice were collected from different place in the state of Colima, Mexico (table 1). The total samples were completed on

four sampling dates in a period from March to September of the year 2019.
Cooked rice was defined as food ready to eat, in its version with or without added vegetables (corn, potato, green bean, carrot and pea), "white" or "red (with tomato puree)". And, pre-cooked or parboiled rice was defined as the preparation that is used as an ingredient to prepare cooked rice or other foods, usually requiring a terminal heat treatment that is usually sublethal on *B. cereus* spores.
Only three samples of parboiled rice contained added vegetables.

Table 1. Distribution of samples by type and date of sampling
Tabla 1. Distribución de muestras por tipo y fecha de muestreo

	Sampling date				
Rice type	**March**	**May**	**July**	**September**	
- Parboiled rice without vegetables	5	6	7	11	**32**
- Parboiled rice added with vegetables	0	1	0	2	
- Cooked rice without vegetables	2	5	11	8	**155**
- Cooked rice added with vegetables	18	28	48	35	

The sample consisted of collecting approximately 100 g of rice, which was deposited in sterile Whirl-Pak™, Nasco™ bags and transported in a cooler (at 4-8 ºC) to the laboratory for analysis in a period not exceeding 2 hours.

Determination of sanitary quality
Sanitary quality was evaluated by observing the counts of aerobic mesophilic bacteria and total coliform bacteria, which were carried out following the official methodology (NOM-092-SSA1-1994: Method for aerobic bacteria count in plate; and NOM-112-SSA1-1994: Determination of Coliform Bacteria).
With base to this, 10 g of the sample to be analyzed in a sterile plastic bag. Was added a 90 mL volume of peptone diluent (Becton™ Dickinson de México) brought to a temperature similar to the sample. I know mixed in Stomacher 400 (Lab System™) for 1 min at normal speed, until obtaining a complete suspension and homogeneous.
The large particles settle, and the amount was transferred desired by taking from the layers upper suspension. They were made from the dilutions corresponding decimals, shaking in a Vortex™ (Scientific™ Inc.).

***B. cereus* count**
The count of *B. cereus* was carried out on MYP agar (mannitol, yolk egg and polymyxin) (Becton™, Dickinson™) by technique surface extension and the plates were incubated at 30 °C /24-48 h (Incubator Termolab™), agree as suggested by the FDA (2012) (21).

Identification of *hblA* and *ces* genes in *B. cereus* strains by PCR
Genomic DNA was obtained by using the Wizard Genomic DNA purification kit (Promega™), following the manufacturer's specifications. For PCR, a reaction mixture was prepared with 14.8 µl of sterile distilled water, 2.5 µl of 10x buffer (supplied with Taq polymerase), 1.3 µl of 50 mM $MgCl_2$, 0.2 µl of deoxynucleoside triphosphates (10 mM) (Invitrogen™), 2 µl of each primer, 1 U of Taq DNA polymerase (Invitrogen™), and 2 µl of a solution containing about 100 ng of DNA. Amplifications were performed on a MultiGene gradient thermal cycler, using primers for the *hblA* (9) and *ces* (22) genes.
For *hblA* amplification, the following conditions were used: 5 min at 94 ºC, 30 cycles of 45 s at 94 ºC, 45 s at 51 ºC, and 1 min at 72 ºC, followed by one cycle of 5 min at 72 ºC (25).
For *ces* amplification, the following conditions were used: 5 min at 94 ºC, 30 cycles of 45 s at 94 ºC, 45 s at 55 ºC, and 45 s at 72 ºC, followed by one cycle of 5 min at 72 ºC (25). A total of 6 µl of PCR product was then loaded onto an agarose gel containing ethidium bromide and visualized under UV light. DNA from the reference strains was used as positive controls; *B. cereus* ATCC
14579 was used for the *hblA* gene, and for the *ces* gene, the NCTC 11143 strain was used.

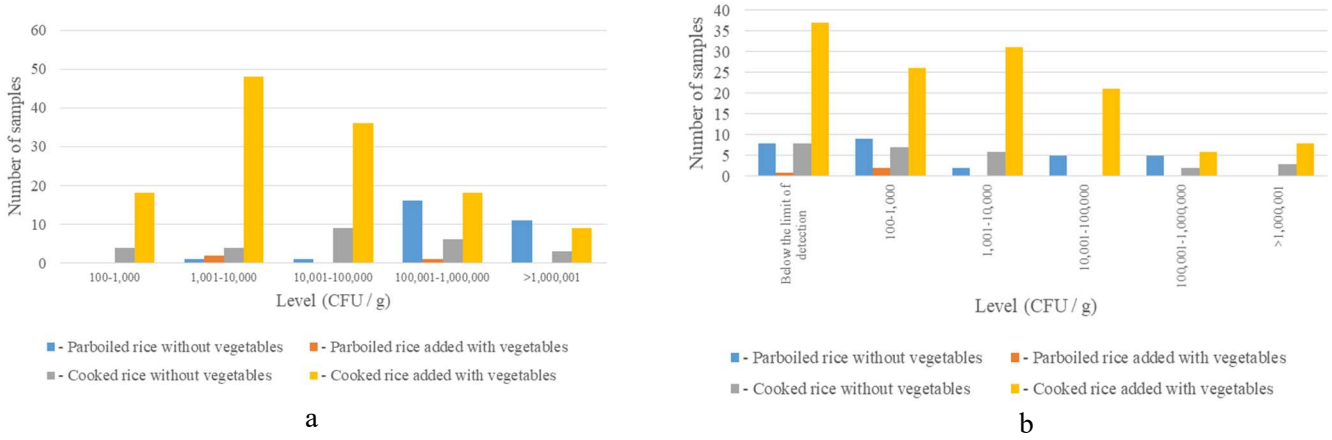
RESULTS AND DISCUSSION

The presence of a high number of mesophilic aerobic bacteria that grow well at or near body temperature means that conditions may have been favorable for the multiplication of pathogenic microorganisms of human or animal origin (23).
Coliform bacteria are frequently used as a bacterial indicator of the sanitary quality of food and water (24). They are a group of bacteria closely related to soil (sowing), water and the intestinal tract of animals, they have been used as indicators of unsanitary conditions

in the production of food and beverages for more than a century. Today, the coliform count is a frequent hygienic indicator in various food and beverage industries. The best-known Coliform, *Escherichia coli*, is a common resident of the intestines of warm-blooded animals, but it can also be found in the natural environment and transmitted to food manufacturing facilities, as well as sources of drinking water.

Rather than looking for a specific species, the coliform test offers a broader view of the organisms in ingredients, finished products, and the overall production environment. In this way, coliforms serve as "indicator organisms", and evidence for their presence in general rather than an individual genus or species may mean that unsanitary conditions exist that can harbor pathogenic organisms. If a sample area is under control, the number of these indicator organisms would be contained in relatively small amounts.

After analyzing the rice samples, it was observed that in 87.5 % of the parboiled rice samples and in 23.2 % of the cooked rice samples, high counts of aerobic mesophilic bacteria were obtained (> 10^5 CFU / g), and in 71 % of the total samples analyzed it was demonstrated the presence of viable total coliform bacteria (figure 1.).



a b

Figure 1. Distribution of mesophilic aerobic bacteria (a) and coliform bacteria (b) in parboiled and cooked rice
Figura 1. Distribución de bacterias mesófilas aerobias (a) y bacterias coliformes (b) en arroz precocido y cocido

In 28.1 % of the parboiled rice samples and in 89 % of the cooked rice samples, *B. cereus* was recovered (table 2).

Table 2. Frequency* of *B. cereus* positivity in cooked or parboiled rice samples
Tabla 2. Frecuencia* de positividad de *B. cereus* en muestras de arroz cocido o precocido

Rice type	*B. cereus*	
	Negative**	**Positive**
- Parboiled rice without vegetables	23	6
- Parboiled rice added with vegetables	0	3
- Cooked rice without vegetables	17	9
- Cooked rice added with vegetables	0	129

* number of samples; ** Not detected

All samples of the cooked or parboiled rice samples containing vegetables (corn, potato, green bean, carrot and pea) showed the presence of *B. cereus*, so these ingredients may be related to the presence of the pathogen in cooked rice or precooked.

Flores-Urban et al. (2014) reported that *B. cereus* was identified in 57% of the 100 analyzed samples of different vegetables (32% of the broccoli samples, 44% of the carrot samples, 84% of the lettuce samples and 68% of coriander samples). Contamination in these vegetables can be present from their origin or during their processing (peeling and / or cutting). Also the process of pregnant in plastic containers under non-sterile conditions could favor the presence of *B. cereus* in the food. In addition to the fact that changes in eating patterns have been adopted, such as the preference for minimally processed fresh foods and increased prevalence of food prepared outside the home, have contributed to the increased incidence of foodborne illness (26).

At least one strain of *B. cereus* was recovered for each positive sample. 162 strains were evaluated by PCR to determine the *hblA* and *ces* genes. The *hblA* gene

(encoding one of the three BL hemolysin subunits) was amplified in 86 % of *B. cereus* isolates, however, no strains carrying the *ces* gene (encoding cereulide) were identified.

CONCLUSION

The inclusion of vegetables as ingredients in the preparation of ready-to-eat or parboiled rice favors the presence of *B. cereus*, which is why it is recommended to improve food preparation practices and if dispensed at the outlet. Because even in figures far below those considered as a health risk, if it is stored in conditions of temperature abuse, as is often the case in the sampled sites, contaminated rice can represent a risk to the health of the consumer.

Conflict of interest:
The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Standardization of an Efficient Technique for the Reporting of CFU/mL in Urine Samples with Bacterial Cell Cytolysis

Estandarización de una técnica eficiente para el reporte de UFC / mL en muestras de orina con citolisis celular bacteriana

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ABSTRACT

Urinary tract infections (UTIs) are common in women due to the anatomy of the urethra and its proximity to the anus.1 Bacterial cell cytolysis (BCC) describes the entry of bacteria into the cells of the UTIs. The most frequent BCC is by Gram-negative bacilli, colonizing, adhering and penetrating cells of the urinary tract. BCC is not considered in the CFU / mL count in urine culture using the conventional procedure. The drawback of the technique is that after BCC an innumerable number of bacteria can be located in a single cell and since the release of intracellular bacteria is not promoted, only a single CFU is quantified and not the real set that remains inside the cell. Such under-notification is significant given that the decision to offer an antimicrobial treatment to the patient depends on the count. This study aims to standardize an efficient technique for reporting CFU / mL in urine samples with BCC. Urine sample was collected. Urochemical profile, urinalysis, cytopathology and a plate count were performed to define the initial amount of bacteria, for later comparison against the post-treatment cell lysis bacterial count. To demonstrate the best cell lysis treatment: a randomized 3x2x3x2 full factorial design was used, in triplicate, whose factors were: Lysis method (Vortex®, Low frequency ultrasound (LFU) and Chemical Buffer), speed and time of exposure, and finally material of the sample container (glass or plastic). The technique that generated the greatest recovery and that reduced the quantification error was; LFU of the sample contained in glass, for 30s. However, regardless of the lysis technique, the use of only one substantially improves (63-734%) the bacterial bacillary recovery of urine samples with bacterial cell cytolysis.

Keywords: Bacterial cell cytolysis, Chemical and mechanical lysis, Low frequency ultrasound

RESUMEN

Las infecciones del tracto urinario (ITU) son frecuentes en mujeres debido a la anatomía de la uretra y su proximidad con el ano.1 La citolisis celular bacteriana (CCB) describe el ingreso de bacterias al interior de las células del TU. La CCB más frecuente es por Bacilos Gram-negativos, colonizando, adhiriéndose y penetrando células de vías urinarias. La CCB no se considera en el recuento de UFC / mL en el urocultivo mediante el procedimiento convencional. El inconveniente de la técnica es que tras la CCB una innumerable cantidad de bacterias pueden situarse en una sola célula y al no promoverse la liberación de las bacterias intracelulares, solo se cuantifica una sola UFC y no el conjunto real que permanecen en el interior de la célula. Tal sub-notificación cobra significancia dado que del recuento depende la decisión de ofrecerle o no un tratamiento

antimicrobiano al paciente. Este estudio pretende estandarizar una técnica eficiente para el reporte de UFC/mL en muestras de orina con CCB. Se colectaron muestra de orina. Se realizó perfil uroquímico, urianálisis, citopatológico y un recuento en placa para definir la cantidad inicial de bacterias, para su posterior comparación contra el recuento bacteriano postratamiento de lisis celular. Para evidenciar al mejor tratamiento de lisis celular: se empleó un diseño factorial completo 3x2x3x2 aleatorizado, por triplicado, cuyos factores fueron: Método de lisis (Vortex®, Ultra sonicavión de baja frecuencia (UBF), Buffer-Químico), velocidad y tiempo de exposición, y finalmente material del contenedor de la muestra (vidrio o plástico). La técnica que mayor recuperación generó y que redujo el error de cuantificación fue; UBF de la muestra contenida en vidrio, durante 30s. Sin embargo, sin importar la técnica de lisis, el sólo empleo de una, mejora sustancialmente (63-734%) la recuperación bacilar bacteriana de muestras de orina con citolisis celular bacteriana.

Palabras clave: Citolisis celular bacteriana, Lisis química y mecánica, Ultrasonicación de baja frecuencia

INTRODUCTION

Urinary tract infections (UTIs) cause approximately 20 million doctor visits. The female gender is the most affected and it is presumed that it can be found in a 10: 1 ratio with respect to the male gender. By definition, UTIs are considered from the moment that the bacteria appear and multiply in the urinary tract beyond the anterior urethra causing classic symptoms of the pathology, in addition, the diagnosis must consider the response of the host, that is, the Epithelial desquamation and immune response (1,2). The migration of bacteria to the bladder is favored by mechanical, physical and chemical factors. To cause an infection, the responsible microorganisms not only need to colonize the urethra, but also adhere to the uroepithelium; these factors are little considered and applied in the different laboratory analyzes (3).

Bacterial cell cytolysis

Bacterial cytolysis is called the phenomenon of introduction of bacteria to cells of the genitourinary tract, the most frequent bacterial cytolysis observed is that caused by gram negative bacilli, colonizing, adhering and penetrating cells of the upper and intermediate urinary tract. The pathogenicity factors involved with this phenomenon are adhesins, lysozymes and cytolysins (1). The activity generated by the various pathogenicity mechanisms present in bacterial genera, develops great cytoarchistructural modifications in uroepithelial cells. These changes can be reversible or irreversible and can be recognized by observing the cellular characteristics found in the urinary sediment. Once the bacteria have recognized the uroepithelial cell receptors, the adhesion process begins, thereby initiating uroepithelial invasion and multiplication; This process occurs due to one of the bacterial genera (pilis, fimbriae, capsule, etc.), so that each uropathogen adopts a different conformation (adherence pattern) characteristic of the epithelium, for which a total of seven different patterns of adherence and bacterial cytolysis, when observing the urinary sediment (2,9).

Problem statement

Leukocyturia encourages bacteria to adhere and subsequently internalize the uroepithelium, establishing and multiplying, a phenomenon recognized as bacterial cytolysis; this event is not considered in the UFC / mL count in the urine culture (3,4). For what we consider that these bacteria that are inside the host cell, must be taken into account, since they significantly influence the final results of the urine culture, since otherwise it is as if a wrong diagnosis was being given (5,8). So far there is no method that is standardized that allows to provide a reliable result, that is, that takes into account the bacteria that are inside the cell in the microbial count.

Purpose of the study

- To evaluate the different methods to release intracellular bacilli in cytolysis, to generate a more efficient count of CFUs.
- Standardize a technique that allows us to extract the bacteria found inside the uroepithelial cells, in order for them to be considered in the microbial count.

Justification and use of the results

The purpose of this work is to demonstrate that when modifying the existing bacterial quantification techniques, a marked increase in the bacterial count (expressed in CFU / mL) is observed in direct relation to the number of cells in bacterial cytolysis, improving the results in the urine culture. Regularly in the quantification of samples with cytolysis there is divergence from one analyst to another, so that the unification of the reporting criteria is essential. To unify the criteria, it is essential to standardize the procedures. Through this design, we will obtain a more efficient method for bacterial counting in samples containing bacterial cytolysis, in order to suggest a more accurate procedure for reporting CFUs found. If

the semi-quantitative method of the general bacteriological examination of urine is considered as the beginning of the diagnosis of urinary tract infections, 70% of the patients would be failing to diagnose, since the phenomenon of bacterial cytolysis masks the development of the real bacterial count. If the release of the bacteria present in the uroepithelium develops and is quantified by modified urine culture, the real diagnosis of UTIs can be made in 92% of patients (using the Kass criteria) (6,7).

MATERIALS AND METHODS

Sampling

The sample of the first morning urine was collected following the medium jet method and homogenized with gentle circular movements avoiding the formation of bubbles and foam.

Urochemical study

Pour 5 mL into 3 sterile tubes of 13 x 100. Mark the sterile tubes with the letters A, B and C. To tube A with 5 mL of urine and using reagent strips for urine determination (Axmilab®) the preliminary characteristics that show a possible infection will be evaluated: Nitrites POSITIVE; Glucose NEGATIVE; Leukocytes +2 (> 75 Cell x 40X field), Bacteria (MODERATE/ABUNDANT); Blood: Traces. Samples with such characteristics will continue to be evaluated.

Cytopathological urinalysis

Tube B should be centrifuged at 2500 revolutions per minute (rpm), for 3 minutes. Once it has been centrifuged, decant 90% of the supernatant, after this step, resuspend the pellet with gentle circular movements in an equivalent volume of Sternheimer-Malbin stain. From tube B: 25 µL of the resuspended is taken and deposited on a clean slide, a coverslip is placed and analyzed under a microscope to verify the presence of cytolysis. Once the presence of cells with bacteria has been identified, a new smear of the sediment is made and later the Gram stain is used. Both preparations already mentioned are observed at 10x to guarantee homogenization and finally the interpretation is carried out at 40x. The presence of urate, oxalate, urea, calcium crystals, as well as squamous cells from the upper, middle or lower pathways should be observed. The search for cells with intracellular bacillary bacteria will be exhaustively described.

Direct count (basal)

A basal count of colony forming units (CFU) was performed on the urine samples that presented cytolysis using Trypticasein Soy Agar (BD-Dixon™). Four plates were inoculated with the aforementioned medium, the first directly; the second with the 1:100 dilution; the third with the 1: 10,000 dilution and the fourth of 1:1,000,000, these were inoculated by means of the surface extension technique. And then the number of CFU / mL of urine was reported.

Post-treatment count

After having the report of each urine sample, those with the highest cytolysis (tube C) are selected and each treatment described below is applied to them. From the initial number of CFU / mL, it is taken as a reference to make the post-treatment dilutions and continue to quantify them with surface extension. A post-treatment gram stain was added to compare the amount of cell lysis and observe the release of intracellular bacilli.

Results analysis plan

Design of experiments: A randomized 3x2x3x2 factorial design will be used in triplicate experimentation. The following table (Table 1) shows the variables and their levels:

Table 1. Variables and levels of the 3x2x3 design of experiments

Tabla 1. Variables y niveles del diseño de experimentos 3x2x3x2

Variable (factors)	Levels
Cell lysis technique	1. Vortex® stirring 2. Low Frequency Ultrasonication (1 W/cm^2) 3. Tris-HCL® Chemical Buffer Lysis
Speed (only for mechanical methods)	1. High 2800 rpm 2. Low 500 rpm
Time (treatment exposure time)	1. 30 s 2. 2 min 3. 5 min
Tube type	1. Glass 2. Plastic.

As response variable: It is the increase in the concentration of CFU / mL in the samples with cytolysis after having submitted them to each of the experiments. Increase in CFU / mL = (Count after lysis experiment / Baseline Direct Count) * 100.

The program to be used for the analysis of the CFU increment values of each procedure performed will be JMP 5.1.0 and thus obtain the best technique to perform in samples that present bacterial cytolysis.

RESULTS AND DISCUSSION

The fact of applying a lysis treatment, either by Vortex, Lysis Buffer and / or Low Frequency Ultrasound (LFU) generated an increase in the count compared to the initial count without lysis treatment. The lysis technique that showed the greatest efficiency in the recovery of the bacterial cells trapped inside the epithelial cells was LFU, followed by Vortex and finally, the lysis buffer. However, statistically there is no significant difference (p> 0.05) between the levels of the type of technique factor, so any technique could be used without discrimination to improve the microbial bacillary recovery of urine samples with cytolysis by 63-734 %. Similarly, no significant difference (p> 0.05) is found between techniques when evaluating them based on the quantification error. Regarding the factor of agitation speed in Vortex® and type of material for LFU, the high speed was the one that showed the greatest increase in the recovery of intracellular bacterial cells with respect to the lower speed. On the other hand, for the type of material used in LFU, the one that showed the greatest recovery of intracellular bacterial cells was glass, showing increases of up to 1273 % with respect to plastic, which showed maximum increases of 832 % over the initial count without LFU. However, no statistically significant difference (P> 0.05) was shown between the levels of the speed factor and type of material used.

CONCLUSION

Regardless of the lysis technique, vortex speed, type of container material used for the LFU and the exposure time in each of the techniques, the simple use of a cell lysis technique substantially improves the recovery of bacteria cells trapped inside invaded epithelial cells (cells with cytolysis). The technique that generates the greatest recovery and that limits the quantification error to a greater degree was the use of LFU from 5 mL of sample contained in a glass tube, for 30 s at room temperature 22-25 ° C. However, since no statistically significant difference is found between the factors and levels tested, it is suggested to use any cell lysis technique interchangeably. It is suggested to continue the research, increasing the treatments and including a more level of technique that combines the lytic chemical power of the Tris buffer solution, vortex agitation and the micro-vibration in LFU generated by glass microbeads, in order to test if better results are obtained in bacterial cell recovery in samples with bacterial bacillary cell cytolysis.

Conflict of interest:
The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Hand Washing Compliance among Retail Food Establishment Workers in Cartagena de Indias, Colombia

Cumplimiento del lavado de manos entre trabajadores de establecimientos minoristas de alimentos en Cartagena de Indias, Colombia

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A B S T R A C T

Inadequate hand washing by food workers is an important contributing factor to foodborne disease outbreaks in retail food establishments (RFEs). We conducted a survey of RFEs to investigate the effect of hand washing training, availability of hand washing facilities, and the ability of the person in charge (PIC) to describe hand washing according to the Ministry of Health and Social Protection on workers' ability to demonstrate food code–compliant hand washing. Only 52% of the PICs could describe the hand washing procedure outlined in the food code, and only 48% of workers could demonstrate code-compliant hand washing. The most common problems observed were failure to wash for 20 s and failure to use a fingernail brush. There was a strong positive association between the PIC being a certified food manager and being able to describe the food code hand washing procedure (odds ratio [OR], 5.5; 95% confidence interval [CI], 2.2 to 13.7), and there was an even stronger association between the PIC being able to describe hand washing and workers being able to demonstrate code-compliant hand washing (OR, 15; 95% CI, 6 to 37). Significant associations were detected among correct hand washing demonstration, physical infrastructure for hand washing, and the hand washing training methods used by the establishment. However, the principal determinant of successful hand washing demonstration was the PIC's ability to describe proper hand washing procedure. These results suggest that improving hand washing practices among food workers will require interventions that address PIC knowledge of hand washing requirement and procedure and the development and implementation of effective hand washing training methods.
Keywords: Hand Washing, Retail Food Establishment, Colombia

R E S U M E N

El lavado de manos inadecuado por parte de los trabajadores de alimentos es un factor importante que contribuye a los brotes de enfermedades transmitidas por los alimentos en los establecimientos minoristas de alimentos (RFE). Realizamos una encuesta de RFE para investigar el efecto de la capacitación en lavado de manos, la disponibilidad de instalaciones para lavarse las manos y la capacidad de la persona a cargo (PIC) para describir el lavado de manos de acuerdo con lo establecido por el Ministerio Colombiano de Salud y Protección Social sobre la capacidad de los trabajadores para que demuestre cómo lavarse las manos en conformidad con el código alimentario. Solo el 52% de los PIC pudo describir el procedimiento de lavado de manos descrito en el código de alimentos, y solo el 48% de los trabajadores pudo demostrar cómo lavarse las manos de acuerdo con el código. Los problemas más comunes observados fueron; no lavarse durante 20 s y no usar un cepillo de uñas. Hubo una fuerte asociación positiva entre el PIC ser un administrador de alimentos certificado y ser capaz de describir el procedimiento de lavado de manos del código de alimentos (razón de probabilidades [OR], 5,5; intervalo de confianza [IC] del 95%, 2,2 a 13,7), y hubo una asociación aún más fuerte entre el PIC puede describir el lavado de manos y los trabajadores pueden demostrar el lavado de manos que cumple con el código (OR, 15; IC del 95%, 6 a 37). Se detectaron asociaciones

significativas entre la demostración correcta de lavado de manos, la infraestructura física para el lavado de manos y los métodos de capacitación en lavado de manos utilizados por el establecimiento. Sin embargo, el principal factor determinante de una demostración exitosa de lavado de manos fue la capacidad del PIC para describir el procedimiento adecuado de lavado de manos. Estos resultados sugieren que la mejora de las prácticas de lavado de manos entre los trabajadores de la alimentación requerirá intervenciones que aborden el conocimiento de PIC sobre los requisitos y procedimientos de lavado de manos y el desarrollo e implementación de métodos efectivos de capacitación en lavado de manos.
Palabras clave: Lavado de manos, Establecimiento minorista de alimentos, Colombia.

INTRODUCTION

Foodborne diseases are a major public health problem in the world (13). Noroviruses are the leading known cause of foodborne illness and are increasingly recognized as the leading cause of outbreaks (1). Infected food workers may transmit norovirus and other foodborne pathogens by touching foods or food contact surfaces with contaminated hands (9, 10). According to the Centers for Disease Control and Prevention, poor personal hygiene is one of the five most common causes of foodborne disease outbreaks (19). Thus, proper and consistent hand washing must be practiced by all food workers to reduce the risk of disease transmission (5, 15, 17). The Ministry of Health and Social Protection (food code) specifies a hand washing protocol for food workers, which includes wetting the hands, applying soap, rubbing the hands together vigorously for at least 20 s, and rinsing with clean water (18). In addition to this, food workers in Cartagena are required to use a fingernail brush during hand washing to scrub areas underneath the nails and between the fingers (18). However, epidemiologic and inspection data show that there is low compliance with hand washing requirements among retail food establishment (RFE) workers (1, 19).

Public health agencies promote hand washing among food workers by requiring that appropriate hand washing facilities be provided in each RFE and that ongoing hand washing training is conducted (18, 20). Thus, all RFEs in Cartagena de Indias, Colombia must have fully equipped hand washing stations, and except for establishments with minimal food preparation, such as those that serve prepackaged food, all must have a state-certified food manager who is trained in safe food preparation, sanitation, and the prevention of foodborne illnesses. Additionally, there must be a designated person in charge (PIC) of a RFE at all hours of operation who is knowledgeable about foodborne disease risk factors, such as poor worker hygiene, and who is responsible for ensuring that appropriate measures are in place to prevent foodborne disease transmission.

Lack of hand washing facilities and ignorance of the health benefits associated with hand washing are important barriers to hand washing in the general population (6, 7, 10, 16). However, the barriers to hand washing in RFEs are not fully understood.

Purpose of the study

We therefore conducted a survey of RFEs to investigate the relationship among hand washing training, the ability of the PIC to describe hand washing according to the food code, and the availability of appropriate hand washing facilities and workers' ability to demonstrate food code–compliant hand washing.

MATERIALS AND METHODS

Data were collected by sanitarians while conducting routine inspections of 123 RFEs. The surveyed establishments included restaurants, delis, bakeries, and grocery stores in 12 inspectional jurisdictions across Colombia. A standardized instrument was used to collect data for the study, and all participating sanitarians were trained to use the instrument before data collection began.

In each establishment surveyed, the PIC was asked if he or she was a state certified food manager. Then the PIC was asked to describe the hand washing procedure stipulated by the food code and to identify which if any of the following methods were used to conduct employee hand washing training: posted materials, training video, use of written training manual, explanation of hand washing requirement and procedure, demonstration of proper hand washing, training with employee sign-off, and/or comprehensive training with formal certification. After the PIC was interviewed, a food handler was asked to demonstrate hand washing according to the food code. Care was taken to select a worker who had not heard the hand washing description given by the PIC. A satisfactory hand washing description or demonstration had to include wetting the hands, lathering soap up to the wrist, rubbing vigorously for 20 s, using a fingernail brush, rinsing with clean water, and drying hands with a disposable paper towel. Following the demonstration, the evaluator conducted an inspection of the establishment's hand washing facilities to determine if they were accessible, supplied with water at a temperature of at least 43 ºC (110 ºF), and clean and if soap, disposable towels, and a fingernail brush were

present at the sink. Inspection of the hand washing facilities was performed after the hand washing demonstration to reduce the likelihood of bias. Statistical analysis of the data was performed with the Statistical Analysis System (SAS) software (SAS Institute, Cary, N.C.) and EpiInfo 2002 (Centers for Disease Control and Prevention, Atlanta, Ga.). The analysis of variance procedure was used to test associations among numerical variables, and associations among categorical variables were tested by X^2 tests.

RESULTS AND DISCUSSION

Training methods

Most establishments provided some type of hand washing training to employees. The number of methods used for hand washing training ranged from no formal training in 14% of the establishments to six different methods in one establishment (Fig. 1). The most frequently reported method used for hand washing training was a verbal explanation of hand washing (Table 1). Among establishments that used only one method of training, demonstration and explanation were the most effective methods in that employees in RFEs that reported using either of these methods were two to three times more likely to demonstrate code-compliant hand washing than were employees who received no formal training (Table 2). However, the effectiveness of all training methods was dependent on the PIC's ability to describe hand washing, and irrespective of the training method, 60 to 84% of workers could demonstrate hand washing when the PIC could describe hand washing and only 0 to 30% of workers could when the PIC could not describe the procedure. There was also a strong positive association between the number of hand washing training methods and worker's ability to demonstrate hand washing (X^2 test for trend, P , 0.01) (Fig. 2).

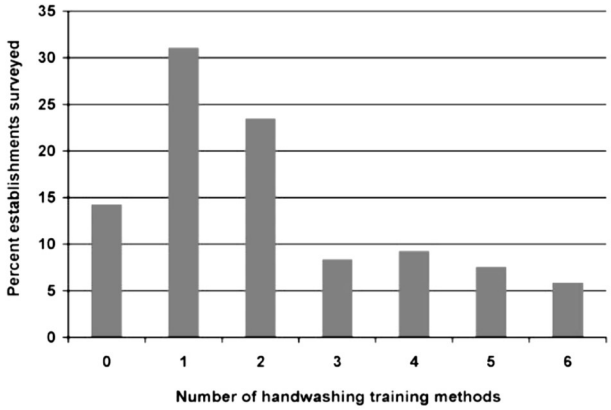

Figure 1. Hand washing training methods used in surveyed establishments. None of the establishments used all seven methods.

Figura 1. Métodos de formación sobre el lavado de manos utilizados en los establecimientos encuestados. Ninguno de los establecimientos utilizó los siete métodos

Table 2. Correct hand washing demonstrations by training method

Tabla 2. Demostraciones correctas de lavado de manos por método de capacitación

Training method	Frequency (%)	No. (%) correct hand washing		Rate ratio
		Yes	No	
Explanation	15	7 (47)	8 (53)	2.2
Demonstration	8	5 (63)	3 (37)	3.0
None	14	3 (21)	11 (79)	Reference
Signs and posters	7	2 (29)	5 (71)	1.4
Training manual	5	1 (20)	4 (80)	1.0
Video	2	0 (0)	2 (100)	0

For 37 establishments that reported using only one hand washing training method compared with 14 establishments that did no formal training. None of these establishments offered formal training with certification.

Table 1. Correct hand washing demonstrations by training methods used

Tabla 1. Demostraciones correctas de lavado de manos mediante los métodos de capacitación utilizados

Training method	Frequency (%)[b]	No. (%) correct hand washing		Rate ratio
		Yes	No	
Explanation	62	37 (60)	25 (40)	2.9
Demonstration	54	32 (59)	22 (41)	2.8
Signs and posters	41	18 (44)	23 (56)	2.1
Sign-off	30	19 (63)	11 (37)	3.0
Training manual	29	19 (66)	10 (34)	3.1
Video	23	12 (52)	11 (48)	2.5
None	14	3 (21)	11 (79)	Reference
Certificate	6	3 (50)	3 (50)	2.4

[a] For all establishments. [b] Fifty-three percent of establishments reported using two or more training methods

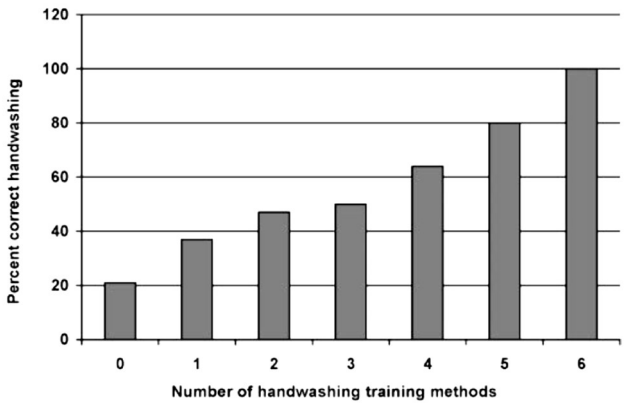

Figure 2. Percentage of correct hand washing demonstrations by number of training methods. None of the establishments used all seven methods.

Figura 2. Porcentaje de demostraciones de lavado de manos correcto por número de métodos de capacitación. Ninguno de los establecimientos utilizó los siete métodos.

Hand washing description

Only 52% of the PICs could describe the hand washing procedure outlined in the food code. The PICs who were state-certified food managers were more likely to be able to describe the food code hand washing procedure (48 [66%] of 73 compared with 8 [25%] of 32; odds ratio [OR], 5.8; 95% confidence interval [CI], 2.3 to 14.7). Failure to specify the need to use a fingernail brush was the most common problem with the hand washing descriptions given by the PICs. However, 77% of PICs who were state certified food managers described the need to use a fingernail brush compared with 38% of uncertified PICs (OR, 5.5; 95% CI, 2.2 to 13.7).

Hand washing demonstration

Only 48% of food handlers could demonstrate hand washing according to the food code. The most frequent problems with the hand washing demonstrations were failure to use a fingernail brush and failure to wash for 20 s. These problems were noted in 89 and 60% of incorrect hand washing demonstrations, respectively. The ability to demonstrate codecompliant hand washing was significantly associated with the PIC being able to describe hand washing (44 [76%] of 58 compared with 9 [18%] of 51; OR, 14.7; 95% CI, 5.7 to 36.5).

Hand washing facilities

Only 68 (55%) of the establishments surveyed were fully equipped for hand washing according to the food code. The most common problems with the hand washing facilities were a lack of fingernail brush and inaccessibility of the hand sink. These problems were noted in 38 and 24% of the establishments, respectively. Hand washing facilities were more likely to be fully equipped in the establishments where a certified food manager was the PIC during the survey (45 [60%] of 75 versus 12 [38%] of 32; OR, 2.5; 95% CI, 1.1 to 6.3). A nailbrush was at the demonstration sink in 62% of establishments, elsewhere in the establishment in 21%, and completely absent in 17% of establishments. If the brush was at the sink it was used 86% (57 of 66) of the time, whereas if it was not at the sink it was used 7% (3 of 44) of the time (OR, 87; 95% CI, 22 to 339).

Poor hand washing by food workers is an important risk factor for foodborne disease outbreaks in RFEs (3, 7, 11, 14, 21). This includes the failure to both wash hands and wash hands correctly. Although we were not able to directly observe the frequency or adequacy of hand washing during routine foodservice operations, we believe that being able to demonstrate proper hand washing technique is a necessary condition for good hand washing practices and may be a useful indicator of likely hand washing compliance in RFEs (13, 17).

Several important findings were made in this study. Among them, we have shown that there is a strong association between the hand washing knowledge of the PIC and the ability of food workers to demonstrate proper hand washing. Assigning a person who is knowledgeable about operational and code requirements to be in charge of a RFE at all hours of operation is essential for ensuring the appropriate detection and resolution of food safety hazards (4).

However, under current Colombian food regulations, a PIC of a food establishment does not have to demonstrate achievement of food safety knowledge standards through testing and certification; thus, the food safety knowledge of individual PICs is highly variable. These results suggest that uncertified managers may lack the skills and/or inclination to ensure appropriate levels of compliance with hand washing by food workers; thus, a certified food manager may need to be present in high-risk RFEs during all hours of operation to help ensure acceptable levels of hand washing compliance.

Workers in establishments that conduct some type of hand washing training could more frequently demonstrate proper hand washing than workers in establishments that did no training. In addition, hand washing performance was directly proportional to the number of methods used to conduct hand washing

training. These findings reinforce the long-held belief that appropriate food safety education can help improve food safety performance in retail establishments (2, 4, 12). In particular, demonstration of hand washing technique and verbal explanations of hand washing appeared to be the most effective training methods when used alone. Both depend on personal communication with the food worker. In contrast, less personal methods, such as the use of training manuals, signs, posters, and videos, did not appear to be successful when used as the sole method. However, these materials may be important to reinforce the primary training. The increasing effectiveness of multiple methods of training also suggests that repeating the training messages in different ways may be important (8).

CONCLUSION

In conclusion, our results suggest that improving hand washing practices among food workers will require interventions that address PIC knowledge of hand washing requirement and procedure, physical facilities for hand washing, and the development and implementation of appropriate training methods. Personal communication with the food worker appears to be important for effective training and is very likely to be important for translating hand washing knowledge into routine practice.

Conflict of interest:
The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Popular Beliefs Towards Chagas Disease in an Endemic Chilean Community

Creencias populares sobre la enfermedad de Chagas en una comunidad chilena endémica

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A B S T R A C T

Chagas' disease remains a major public health concern throughout much of Latin America. In Chile, segments of the population experience *Trypanosoma cruzi* infection rates as high as 65%, indicating that control programs are still needed. Few data are available concerning people's health beliefs related to Chagas' disease in heavily infected populations. Such health beliefs may significantly impact the effectiveness of intervention schemes. The purpose of this study was to assess health beliefs related to Chagas' disease in a population experiencing high infection rates with the causal parasite. The focal population for the study consisted of the residents of Paine, a rural community in Maipo, Metropolitan region of Santiago, Chile. The results indicate that a majority of the population had a high degree of knowledge about Chagas' disease and the vector involved in its transmission. These findings indicate that control programs conducted by the Chilean Ministry of Health have included effective educational components.

Keywords: Knowledge, Attitudes, Practice; *Trypanosoma cruzi*; Chagas Disease

R E S U M E N

La enfermedad de Chagas sigue siendo un importante problema de salud pública en gran parte de América Latina. En Chile, segmentos de la población experimentan una tasas de infección por *Trypanosoma cruzi* de hasta 65%, lo que indica que aún se necesitan programas de control. Hay pocos datos disponibles sobre las creencias de salud de las personas relacionadas con la enfermedad de Chagas en poblaciones muy infectadas. Tales creencias sobre la salud pueden tener un impacto significativo en la efectividad de los esquemas de intervención. El propósito de este estudio fue evaluar las creencias de salud relacionadas con la enfermedad de Chagas en una población que experimenta altas tasas de infección con el parásito causal. La población focal para el estudio consistió en los residentes de Paine, una comunidad rural en Maipo, región Metropolitana de Santiago, Chile. Los resultados indican que la mayoría de la población tenía un alto grado de conocimiento sobre la enfermedad de Chagas y el vector involucrado en su transmisión. Estos hallazgos indican que los programas de control llevados a cabo por el Ministerio de Salud de Chile han incluido componentes educativos efectivos.

Palabras clave: Conocimiento, Actitudes, Práctica, *Trypanosoma cruzi*, La enfermedad de Chagas

INTRODUCTION

Chagas' disease is an extreme consequence of infection with the parasitic protozoan *Trypanosoma cruzi*. The disease continues to be a major public health concern throughout Latin America, despite numerous control and eradication programs. It has been estimated that between 16 and 18 million people are infected with *T. cruzi and* that 100 million more individuals are at risk of infection (16). T. cruzi is transmitted to humans by several genera of triatomine bugs, including *Triatoma, Rhodnius,* and *Panstrongylus* (14). Transmission occurs subsequent to the blood meal, when feces deposited by infected bugs come into contact with open wounds or mucous membranes (14).

Houses with thatched roofs and/or mud walls provide a multitude of sites for the triatomine bug vector to

inhabit, and evidence of infestation of these types of dwellings in Latin America dates to prehistoric times (8,5,9). *T. cruzi* has a broad range of hosts, including such domestic animals as goats, sheep, guinea pigs, dogs, and cats (8,9). Chickens, while not a host of *T. cruzi*, provide a source of blood meals for the triatomine bugs and are commonly kept in the yards of houses in the study area. Keeping such domestic animals in close proximity to the house may encourage proliferation of the household bug population.

Intensity of bug infestation has been shown to be directly correlated with risk of *T. cruzi* infection in household residents (17). Epidemiological patterns of *T. cruzi* infection have been suggested to reflect variation in household risk factors (12).

Chagas' disease generally occurs in two phases. First, there is an acute phase lasting 2-3 months following parasitic infection, with possible formation of a characteristic swelling or chagoma at the infection site (14). Many individuals remain asymptomatic throughout the acute phase of the illness, although between 5% and 10% of individuals experience severe, sometimes fatal, disease during this phase (14). About 40% of those infected recover spontaneously from the acute phase and remain seropositive but disease-free for the remainder of their lives. In the remaining 60% of cases the disease enters a quiescent period lasting as long as 30 years, until the onset of the chronic phase, which can be characterized by progressive cardiomyopathy, megacolon, and/or megaesophagus (11,14).

There are currently no prophylactic drugs available to prevent infection with *T. cruzi* (4). Chemotherapy for *T. cruzi* infection has centered on the use of two nitroarenes, Nifurtimox and Benznidazole. These drugs are effective in decreasing early parasitemia, but their utility during the chronic phase of Chagas' disease is questionable (8,4). Both drugs have severe side effects and are carcinogenic in rabbits (8,21,22,23). Despite extensive biomedical research on Chagas' disease, relatively little information has been generated regarding people's perceptions of the disease, T. cruzi infection, or the relative importance of this disease in endemic communities. A survey of Mambaí, in the State of Goiás, Brazil, where 33% of individuals were seropositive and 32% of houses were infested with the vector, revealed that most individuals had knowledge of both the vector and the characteristics of Chagas' disease (2). Almost everyone reported that the bugs were the cause of the disease, 84% of individuals understood the type of disease transmitted by the vector, and 56% recognized that control of bug infestation was important. At the time of the study, Mambaí had been the focus of a long-term research project on Chagas' disease and was also the site of a regional health post. This study suggested that the Brazilean public health programs had been successful in their educational efforts. In Maipo we also applied their survey in Paine a rural community that lacked a health post. Similar to the situation in Mambaí, Brazil most individuals that were interviewed agreed that triatomine bugs were harmful and transmitted disease. However, only 55% of the residents of the rural community were knowledgeable of the characteristics of Chagas' disease. Despite decreased awareness of the signs and symptoms of Chagas' disease in this community as compared to Mambai, 71% of individuals recognized the importance of bug control in diminishing disease rates. Even in the absence of a local health post, control programs had a significant impact on transmission of information about the Chagas' disease vector. Conversely, a recent study in Guatemala focusing on an area with a seropositivity rate of approximately 11% suggested that none of the residents knew of Chagas' disease as a specific disease entity (13). No signs or symptoms described as resulting from bites by triatomine bugs were associated with Chagas' disease (13). However, many people were aware of the bugs, which they considered a household nuisance (13). High rates of recognition of the role of triatomine bugs in Chagas' disease as reported by Bizerra and colleagues (2) stand in contrast to many reports on knowledge and attitudes for other vector-transmitted diseases. For example, malaria studies have frequently shown a lack of accurate knowledge about disease etiology in high-risk areas. A survey conducted in five West African communities showed that only 25-50% of residents identified mosquitoes as the cause of malaria (1). A survey of residents in a coastal plain area in Guatemala found that while 93% of individuals recognized that a mosquito which had previously bitten a malaria patient could transmit the disease, a variety of other causes were believed to exist (19). Fifty percent of residents thought that houseflies could also transmit the disease, 77% thought malaria could result from drinking unboiled water, 77% felt that bathing too frequently could result in malaria, and 62% implicated lack of sleep as a causal factor (19). Lymphatic filariasis is another mosquitoborne disease which has been the focus of large-scale intervention programs. As reviewed by Evans *et al.* (1993), studies of local knowledge of disease causation in endemic

communities have generally shown a variety of alternative causal explanations (7). Filariasis has been attributed to contact with cold water (20), consumption of contaminated foods or drink (3,10), and aggravation of physical injury (3). A survey in a Malaysian community where 6% of individuals were infected with *Brugia malayi* found that only 8% of individuals identified the mosquito as the cause of filariasis, and 42% responded that they could not identify the cause of filariasis (10). Likewise, in an endemic area of Tahiti which had been the focus of an antifilarial campaign for over 25 years, only 13% of filariasis patients identified mosquitoes as the cause of infection as compared to 41% who attributed the disease to ankle injuries (3). A study of an endemic area in Haiti showed that only 13% of residents who recognized filariasis attributed the disease to mosquito transmission, while 48% attributed the disease to contaminated soil or water (6). Onchocerciasis is a filarial infection transmitted by blackflies (15). A survey of attitudes towards this disease among heads of households in an endemic area of Guatemala showed that only 50% of individuals recognized the role of an insect bite in disease transmission (18). Other purported causes included consumption of poorquality foodstuffs or water and physical injury (18). The literature suggests that knowledge of vector transmission is frequently poor in areas endemic for vector-transmitted diseases. A high prevalence of alternative explanations for disease causation has significant implications for the potential efficacy of intervention programs (7,1) Local beliefs about disease causation should be assessed to determine if public health education programs are effective, and such evaluation should be performed periodically to ensure that the quality of education is maintained and that accurate information is being appropriately transferred to the at-risk population.

Purpose of the study

The purpose of this study was to assess local attitudes and beliefs about Chagas' disease in an endemic area of Metropolitan region of Santiago, Chile. This area has been the focus of an active Chagas' disease control program by the Chilean Ministry of Health for over a decade.

MATERIALS AND METHODS

We interviewed adults representing 59 households located in the Paine area, which is endemic for Chagas' disease. Over 60% of adults in this farming county are positive for infection with *T. cruzi* (24). Active transmission of *T. cruzi* infections still occurs, despite insecticide spraying and homebuilding improvement programs which have been operating in the area for the last 14 years (21).

A recent survey of 1,931 individuals from the region indicated that 5% of children aged 10 years or under, 17% of individuals between the ages of 10 and 15 years, 29% of individuals between the ages of 15 and 21 years, and 61% of individuals over 21 years were seropositive for *T. cruzi* (21, personal communication). An interview guide was used to elicit general health concerns and attitudes towards Chagas' disease in particular.

All interviews were conducted in Spanish at the homes of the sampled individuals. Nineteen men and forty women, each living in a different household, were sampled in a house-to-house survey of 4 villages in the area.

The population is an admixed population with European, African, and Amerindian genes represented. Sampled individuals ranged in age from 20 to 79 years, with a mean age of 38.5 years. Individuals were asked about the most important health problems in Paine, whether or not Chagas' disease is an important health problem, the causes of Chagas' disease, symptoms of Chagas' disease, treatments for Chagas' disease, and presence of triatomine bugs in and around the household.

RESULTS AND DISCUSSION

Perceived Health Problems in Paine

Individuals were asked to identify the most important health problems for people residing in the Paine area. Fever was the most frequently mentioned health problem, identified by 50.8% of the sampled individuals. Colds and flu were mentioned as major health problems by 49.2% of the sample, and headache was identified as a major health problem by 47.5% of sampled individuals. Chagas' disease was identified spontaneously by 45.8% of the sample as being a major health problem for residents of the Paine area. Other commonly mentioned health problems included stomach ache (28.8%), heart problems (15.3%), back problems (13.6%), and diarrhea (11.9%). We then asked specifically if Chagas' disease was an important health problem in the Paine area, and 86.4% of the sample replied in the affirmative, often noting that they knew many people who had the disease. Seven individuals did not consider Chagas' disease an important health problem in the area, and one person

did not know whether or not the disease was a major concern in the region.

Causes of Chagas' Disease

Responses regarding causation of Chagas' disease are summarized in Table 1. When asked how an individual acquires Chagas' disease, 72.9% reported that one gets the disease from the barbeiro (triatomine bug vector). Thus, the majority of individuals were aware of the vector involved in the disease transmission.

Several other causes of Chagas' disease were also reported, including bad water (11.9%) and mosquitoes (3.4%). One individual attributed the cause of Chagas' disease to the insecticide used to kill the triatomine vectors. Twelve individuals (20.3%) responded that they did not know the cause of Chagas' disease or how one could catch it. One person suggested that the disease was familial, an observation consistent with a recent genetic study of susceptibility to infection with *T. cruzi* (24).

Table 1. Causes of Chagas' disease as reported by 59 study participants

Tabla 1. Causas de la enfermedad de Chagas según lo informado por 59 participantes del estudio

Cause	# of Respondents	% of Respondents
Triatomine bug vector	43	72.88
Bad water	7	11.86
Mosquito	2	3.39
Insecticide	1	1.69
Hereditary	1	1.69
Don't know	12	20.34

Symptoms of Chagas' Disease

The majority of individuals interviewed had accurate knowledge of the symptoms experienced by individuals with Chagas' disease. Symptoms identified by respondents included chest pain (42.4%), respiratory problems (20.3%), fatigue (35.6%), leg pain (35.6%), nervousness (16.9%), swelling (15.3%), dizziness (13.6%), headache (11.9%), digestive problems (11.9%), general body pain (10.2%), high blood pressure (8.5%), general weakness (6.8%), stomach pain (6.8%), gas and bloating (6.8%), insomnia (5.1%), and rapid heart beat (5.1%). The range of symptoms correctly identified includes signs of cardiomyopathy and signs of gastrointestinal involvement. Only ten individuals (16.9%) were unable to identify any symptoms associated with Chagas' disease.

Treatment of Chagas' Disease

A substantial proportion of the sample (25.4%) reported that they did not know if there was a treatment available for Chagas' disease, and 10.2% of those interviewed asserted that no treatment existed. Sixty-four percent indicated that medicine for treatment of Chagas' disease was available, although over half of these individuals said that the medicine was only effective for recently acquired disease. Responses regarding treatment of Chagas' disease are outlined in Table 2. The responses accurately reflect the lack of an efficacious drug therapy for Chagas' disease during the chronic phase.

Table 2. Treatments for Chagas' disease reported by 59 study participants

Tabla 2. Tratamientos para la enfermedad de Chagas informados por 59 participantes del estudio.

Treatment	# of Respondents	Percentage of Respondents
Medicine	13	22.03
Medicine, but only for newly acquired disease	25	42.37
No treatment exists	6	10.17
Eliminate bugs	1	1.69
Don't know of any	15	25.42

Presence of Triatomine Bugs in the Household

The success of insecticide spraying programs is indicated clearly by the fact that 95% of sampled individuals reported that they did not have triatomine bugs in the house. However, 39% of those who did not currently have bugs in the house reported that they had seen them in the household frequently prior to initiation of the insecticide spraying programs.

The results indicate that residents of the region of Paine have a high level of knowledge and awareness of Chagas' disease and the triatomine vector responsible for transmission of the disease. People who were interviewed readily differentiated between "Chagas' of the blood", (seropositivity) which did not necessarily result in illness, and Chagas' disease which could involve a broad range of gastrointestinal or cardiac symptoms. The triatomine vector was spontaneously identified as the cause of Chagas' disease by the majority of respondents.

These results support those of Bizerra *et al.* (1981) and stand in contrast to the majority of results reported for vector-transmitted diseases (2). We feel several reasons account for this high level of knowledge about Chagas' disease and the role of the triatomine vector in its transmission. First, the insect vector is relatively large

in size, and the bite can be quite painful. Both the insect and the bite are more noticeable than for other smaller disease vectors such as mosquitoes. Additionally, levels of infestation of the houses were very high prior to initiation of the insecticide control programs.

CONCLUSION

Individuals in the study reported seeing large numbers of bugs and being bitten frequently prior to the intervention by the Chilean Ministry of Health's programs aimed at controlling the insects. These programs are all household-based and involve spraying of the house by Ministry of Health workers. Workers wear protective clothing and instruct household members about special precautions for avoiding contact with the insecticide immediately after spraying. Thus the insecticide control program is highly visible and has a clear and direct impact in substantially diminishing the bug population inside households. Ministry of Health personnel informally convey information about Chagas' disease and the role of the bug in transmission of disease during visits for insecticide spraying of the houses. This educational process appears to have been highly effective in establishing the link between the vector and the disease and in disseminating information about the signs and symptoms of the disease. In our experience, whenever bugs were found in the household they were collected and saved for later inspection by Ministry of Health personnel. The Chilean Ministry of Health has focused considerable effort on the control of Chagas' disease in the Paine area. The results of our survey indicate that these programs have effectively disseminated information about Chagas' disease and its vector to the atrisk population.

Conflict of interest:
The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Nisin as Biopreservatives against *Listeria* spp. in Meat Products

Nisina como biopreservantes contra *Listeria* spp. en productos cárnicos

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A B S T R A C T

The antilisterial effect of nisin A and Z in meat and meat products (cooked ham, minced pork meat, deboned chicken breasts, pate, and slightly fermented sausages [espetec]) have been shown. An infective dose of 5 to 10 most probable numbers (MPN)/g to simulate the counts of *Listeria* generally found in meat products was used. Nisin at 4,800 AU/g reduced the numbers of *Listeria innocua* by 7.98 log cycles in cooked ham and by 9 log cycles in pate when stored at 78C for 37 days. In deboned chicken breasts stored at 7ºC for 7 days, 4,800 AU/cm^2 of nisin diminished the *L. innocua* counts in 5.26 log cycles when compared to the control batch. In minced pork meat held at 7ºC for up to 6 days, 1,600 AU/g kept *L. innocua* counts under 3 MPN/g, while the control batch reached 50 CFU/g. In espetec sausages, 648 AU/g diminished the number of *L. innocua* under 50 CFU/g from the fifth day until the end of the process (12 days) while the control batch kept the initial counts (3 x 10^4 CFU/g).

Keywords: Nisin, Meat processed products, *Listeria* spp., Biopreservatives

R E S U M E N

Se ha demostrado el efecto antilisterial de la nisina A y Z en carnes y productos cárnicos (jamón cocido, carne de cerdo picada, pechugas de pollo deshuesadas, paté y salchichas ligeramente fermentadas-espetec). Se utilizó una dosis infecciosa de 5 a 10 números más probables (MPN) / g para simular los recuentos de *Listeria* que se encuentran generalmente en productos cárnicos. La nisina a 4.800 AU / g redujo el número de *Listeria innocua* en 7.98 ciclos logarítmicos en jamón cocido y en 9 ciclos logarítmicos en paté cuando se almacenó a 7°C durante 37 días. En pechugas de pollo deshuesadas almacenadas a 7ºC durante 7 días, 4.800 AU / cm^2 de nisina disminuyeron los recuentos de *L. innocua* en 5.26 ciclos logarítmicos en comparación con el lote de control. En carne de cerdo picada mantenida a 7ºC por hasta 6 días, 1,600 AU / g mantuvieron los conteos de *L. innocua* por debajo de 3 MPN / g, mientras que el lote de control alcanzó 50 UFC / g. En embutidos espetec, 648 AU / g disminuyó el número de *L. innocua* por debajo de 50 UFC / g desde el quinto día hasta el final del proceso (12 días) mientras que el lote control mantuvo los recuentos iniciales (3 x 104 UFC / g).

Palabras clave: Nisina, Productos cárnicos procesados, *Listeria* spp., Bioconservantes

INTRODUCTION

The ingestion of products contaminated with *Listeria monocytogenes* is a health threat to a high-risk population such as the immunocompromised, children, pregnant women, and senior citizens. *Listeria* has been involved in 0.7, 1.7, 7.4, and 16.8 cases per million of the foodborne infections in Spain, Canada, the United States, and France, respectively, and an average of 40% was fatal (6, 12, 15, 38). The last outbreak was in the United States; from August to December of 1998, 75 cases of listeriosis distributed in 11 states were detected and 20% of the patients died (10). The percentage of *Listeria* among the food products assayed in several countries was very variable. In pork meat products, the percentage of *Listeria* was 3.1% in carcasses, 7 to 58% in raw meat, 9.6 to 58% in minced meat, 15 to 100% in raw sausages, and 10 to 35% in cooked meat products (36). In general these products contained a low number of *Listeria*, less than 5 CFU/g, although 10^4 to 10^5 CFU/g were found in pate (7, 32, 33). In poultry, the percentage of positive samples for *L. monocytogenes* ranged from 23 to 85%, and the counts were below 10^2 CFU/g (26, 46). The control of *L. monocytogenes* in foods is difficult due to its ability to grow at a wide

range of temperatures (3 to 45ºC), its tolerance to pH (4.8 to 9.6), to high sodium chloride levels (<10%), sodium nitrite (<1,000 ppm), and its resistence to 10 ppm of the majority of disinfectants that are inhibitory to other food pathogens (7, 13, 14, 43). As common control parameters alone will not preclude microbial hazard by *L. monocytogenes* and as consumers demand more natural products, alternative control measures like biopreservation (such as bacteriocinogenic lactic acid bacteria cultures, their bacteriocins, or both), reducing storage temperature to <2ºC, and postprocessing pasteurization have received increasing attention.

Several authors have described the use of bacteriocinproducing starter cultures to inhibit the outgrowth of *L. monocytogenes* in raw meat, German-style fresh sausages, American-style fermented semidry and dry sausages, Italian salami, Spanish dry fermented sausages, frankfurter sausages, and in vacuum-packaged wieners (4, 5, 8, 11, 12, 16, 21–23, 28, 29, 34, 37, 39, 45, 47, 48). Nisin is a bacteriocin produced by a group of Gram-positive bacteria that belongs to *Lactococcus* and *Streptococcus* species.

Nisin is classified as a Type A (I) lantibiotic that is synthesized from mRNA and the translated peptide contains several unusual amino acids due to post-translational modifications. Over the past few decades, nisin has been used widely as a food biopreservative. Since then, many natural and genetically modified variants of nisin have been identified and studied for their unique antimicrobial properties. Nisin A and Z is an FDA approved and GRAS (generally regarded as safe) peptide with recognized potential for eating. Over the past two decades the application of nisin has been extended to industrial and biomedical fields.

Purpose of the study

The aim of this study was to show the antilisterial activity of nisin A and Z produced by *Lactococcus lactis* ATCC 7962 in slightly fermented sausages (espetec), cooked ham, pate, minced pork, and deboned chicken breasts.

MATERIALS AND METHODS

Bacterial cultures and media

Lactococcus lactis ATCC 7962 (nisin A y Z producer) (3, 35) was incubated in MRS broth at 30ºC. *Listeria innocua* CTC1014 (nisin A and Z indicator in the bacteriocin titer assays) (21) was grown in tryptic soy broth with 0.6% yeast extract at 30ºC. *Lactobacillus sake* FVM 148 (nisin Z indicator in the bacteriocin titer assays) (9, 40) and *Pediococcus pentosaceus* FBB63 (nisin Z indicator in the bacteriocin titer assays) (9, 19) were incubated in MRS at 30ºC. Preparation of bacteriocin. *Lactococcus lactis* ATCC 7962 was grown for 18 h at 30ºC in MRS broth. The cells were removed by centrifugation at 5,000 x g for 10 min at 4ºC. The supernatant fluid was collected and the bacteriocins (nisin A and Z) were pre cipitated by adding 300 g/liter of ammonium sulfate. After 30 min incubation at 0ºC, the precipitated proteins were collected by centrifugation at 8,000 x g at 4ºC for 30 min. The precipitates were dissolved in 3 mM sodium phosphate buffer at pH 7.0 and heated at 80ºC for 10 min. The concentrated bacteriocins were stored at -80ºC. A modification of the critical dilution assay (42) was used to determine the bacteriocin titer (21).

Inoculum preparation

Listeria was grown overnight in tryptic soy broth with 0.6% yeast extract at 30ºC. Cells were harvested by centrifugation (5,000 x g for 10 min), resuspended in tryptic soy broth with 0.6% yeast extract and glycerol (50%), and stored at -40ºC. Viable counts were obtained in tryptic soy agar with 0.6% yeast extract. For food application, cells were diluted in 0.85% NaCl when necessary. *L. innocua* CTC1014 was used instead of *L. monocytogenes* CTC1011 in order to avoid dissemination of the pathogen in the pilot plant (21). Previous assays showed that *L. monocytogenes* was more sensitive to nisin A and Z than *L. innocua* (2, 9).

Product manufacture and experimental design: slightly fermented sausages (espetec)

A shoulder/belly proportion of 60 to 40 was ground in a meat grinder by passing through a 6-mm plate (Tegmag, Barcelona, Spain) and mixed with the additives (g/kg): NaCl, 22; KNO_3, 0.15; $NaNO_2$, 0.15; sodium ascorbate, 0.5; sodium citrate, 3; dextrose, 2; milk powder, 20; lactose, 30; casein, 5; black pepper, 2; red cochineal, 0.02; and water, 60 (Sanofi Bio-Industries S.A., Barcelona, Spain). The mixture was vacuum mixed for 3 min in a vacuum blender (Tegmag). The sausages were vacuum stuffed using a continuous stuffing machine (Tecnotrip, Terrassa, Spain) and a 40- to 42-mm-diameter pork intestine casing. The fresh weight of each sausage was 284 g (±27 g). After dipping in a solution of *Penicillium nalgoviensis* (PNT1- NG8, Rhone-Poulenc Texel S.A., Dange Saint Roman, France), they were placed in a controlled room. The conditions during the process were as follows: 16ºC and 72% relative humidity for 4 h to dry the casings, 15ºC, and 85% relative humidity

for 5 days in order to facilitate the growth of the microflora, and 13ºC and 80% relative humidity up to 12 days (18). Three independent sausage treatments were conducted. Batch I, without added bacteriocin; batch II, with 221 AU/g of nisin A and Z; batch III, with 648 AU/g. *Listeria* was added together with the additives at 10^4 CFU/g. Triplicate samples were taken at selected times (0 h, 1 h, and 1, 2, 3, 4, 5, 6, 7, 9, and 12 days of processing).

Cooked ham. Skinless and deboned fresh ham pork (15%) was injected with a brine solution containing per g/kg: NaCl, 18; NaNO2, 0.1; dextrose, 5; carrageenan, 1; and water, 100 by a multineedle Inject Star BI13B (Dordal SA, Barcelona, Spain). The injected fresh ham was equilibrated for 48 h at 4ºC by alternating tumbling (1 h per day) in a stainless steel vacuum massaging drum (Dordal SA). The injected ham was canned and cooked at 70ºC until the internal temperature reached 67ºC (approximately 8 h) (Unimatic oven UM1/1500, Doleschal, Steyr, Germany). After cooling it was sliced. Three independent lots were prepared. Batch IV, without added bacteriocin; batch V, with 1,600 AU/g of nisin A and Z; batch VI, with 4,800 AU/g. The slices were inoculated with Listeria (5 most probable number [MPN]/g) spread over the slice surface. Afterward, appropriate nisin concentrations were also spread over the surface of each slice. The application of Listeria and bacteriocin added a maximum volume of 10 ml/g in order not to change the damp of the slices. The slices were stored at 7ºC for 37 days in sterile plastic boxes. Triplicate samples were taken at selected times (0, 1, 4, 10, 18, and 37 days of storage).

Pate

Pork liver (250 g) was ground, brined for 18 h in a solution containing in g/kg: $NaNO_2$ (4.2) and mixed with sodium chloride (7.5), white pepper (1.0), paprika (5.0), onions (20), garlic (5), and liquid whole eggs (50), and homogenized in a cutter at 5 to 8ºC. Milk (250) and emulsified fat (450) at 55ºC were added to the mixture and homogenized with the rest of the ingredients in a three-blade cutter (CM-22, Mainca, Granollers, Spain) to obtain a fine-ground product. The product was stuffed and cooked at 75ºC until the internal temperature reached 728C (approximately 2.5 h in a Unimatic oven, UV 1/1500), cooled down in a refrigerator for 24 h, and sliced. *Listeri*a was spread over the surface at 5 MPN/g. In the appropiate batches, bacteriocin were later applied and spread over the surface. The application of *Listeri*a and nisin added a maximum volume of 10 ml/g in order not to change the damp of the slices. Three independent treatments were done: batch VII, without added bacteriocin; batch VIII, with 1,600 AU/g of nisin A and Z; batch IX, with 4,800 AU/g. The product was stored at 7ºC for 37 days in sterile plastic boxes. Triplicate samples were taken at selected times (0, 1, 4, 10, 18, and 37 days of storage).

Minced pork meat

Raw lean pork shoulder and belly fat (50:50) were ground through a 6-mm plate and mixed with *Listeria* at 5 MPN/g for 5 min in a vacuum blender (Tecmag). Afterward, nisin were added in the appropiate batches and the whole mix was vacuum blended during 5 more minutes. The application of *Listeria* and nisin added a maximum volume of 15 ml/g. Four different treatments were prepared: batch X, without added bacteriocin; batch XI, with 1,600 AU/g of nisin A and Z; batch XII, with 3,200 AU/g; and batch XIII, with 4,800 AU/ g. The product was stored at 7ºC for 6 days in sterile plastic boxes. Triplicate samples were taken at selected times (0, 1, 2, 4, 5, and 6 days of storage).

Chicken breasts

Skinless and deboned chicken breasts were selected after slaughtering and three different treatments were conducted: batch XIV, nonadded bacteriocin; batch XV, with 3,200 AU/cm^2 of nisin A and Z; and batch XVI, with 4,800 AU/ cm^2. *Listeria* was spread in all the batches over the surface of the chicken breast with a glass spreader at 10 CFU/cm^2. Nisin were spread afterward in the adequate batches. A maximum volume of 10 ml/g was used for the inoculum of *Listeria* and nisins. The product was stored at 7ºC for 7 days in sterile plastic boxes. Triplicate samples were taken at selected times (0 h, 1 h, and 3 and 7 days of storage).

Physicochemical evaluation

Water activity of the different products assayed was measured with an aw-cryometer (awk-10, Nagy, Ga¨ufelden, Germany). pH was measured with a penetration electrode (Crison Instruments, Barcelona, Spain). The percentage of fat was evaluated by the standard protocol ISO-1443 and total nitrogen content by ISO R-937. The nonprotein nitrogen (NPN) and nitrogen a-amino (N-a-amino) was obtained by standardized protocol no. 24.046 (1). The sodium chloride content was determined by the colorimetric determination of Zall *et al.* (49) adapted to the segmented continuous flow autoanalyzer (Technicon Ltd., Dublin, Ireland). Triplicate samples were taken at

the beginning of the experiments, unless indicated. *Espetec* was evaluated at the end of the manufacturing process.

Microbiological sampling

Triplicates from each treatment were sampled at selected times to determine *Listeria* counts. Ten grams of each sample were mixed (1:10) with dilution medium (0.1% peptone and 0.85% NaCl or *Listeria* enrichment broth (UVM1, Oxoid Division, Unipath Co., Ogdensburg, N.Y.) and placed in a stomacher bag (model 400, Cooke Laboratory Products, Alexandria, Va.) for 1 min. For chicken breasts, an area of 39.3 cm^2 was sampled by the swab method. *Listeria* was enumerated by the spread plate technique in Palcam agar (Merck) at 30ºC for 48 h (44). When the counts of *Listeria* were lower than 10^2 CFU/g or cm^2, the MPN technique using nine tubes (three per dilution, 21, 22, 23) of *Listeria* enrichment broth (UVM1, Oxoid) incubated at 37ºC during 24 h were used. Positive tubes were confirmed by streaking 10 ml in Palcam agar and incubated at 30ºC for 48 h (30). *Listeria* grows as gray–green-colored colonies with a black zone.

Table 1. Physicochemical characteristics of the meat products used[a]
Tabla 1. Características fisicoquímicas de los productos cárnicos empleados[a]

Product	pH	NaCl	a_w	Fat	NMP/N_{total}	*N*-α-amino/N_{total}
Espetec-fermented sausages	6.06 ± 0.01	3.5 ± 0.01	0.87 ± 0.01	41.50 ± 0.32	6.20 ± 0.20	1.86 ± 1.41
Cooked ham	6.36 ± 0.01	2.02 ± 0.06	0.98 ± 0.01	3.48 ± 0.17	8.58 ± 0.20	0.89 ± 0.71
Pâté	6.34 ± 0.00	1.79 ± 0.01	0.97 ± 0.00	20.01 ± 0.16	23.97 ± 0.10	2.79 ± 0.71
Minced meat	5.90 ± 0.01	0.11 ± 0.00	0.99 ± 0.01	13.09 ± 0.13	13.01[b] ± 0.02	1.49[b] ± 0.17
					14.83[c] ± 0.03	1.70[c] ± 0.00
Chicken breast	5.78 ± 0.00	0.19 ± 0.00	1.00 ± 0.00	1.36 ± 0.08	9.97[b] ± 0.1	0.85[c] ± 0.71
					27.80[c] ± 0.06	2.71[c] ± 0.00

[a] Values are the mean of triplicates plus its standard deviation. The values of NaCl, fat, NMP/Ntotal, and N-a-amino/Ntotal are in percentages. [b] Beginning of the storage period. [c] End of the storage period.

RESULTS AND DISCUSSION

Five different types of meat and meat products (espetec, cooked ham, pate, minced pork meat, and chicken breasts) were assayed in order to evaluate the antilisterial effect of bacteriocin. At selected times during storage, samples were taken in triplicate to determine the *L. innocua* CTC1014 counts. In espetec, *L. innocua* CTC1014 grew from 3.2 x 10^4 CFU/g to 2 x 10^5 CFU/g after 4 days of processing and decreased down to the initial counts at the end of the process (12 days) in the control batch without bacteriocin. In batch II inoculated with 221 AU/g of nisin, *Listeria* counts diminished from 3.2 x 10^4 CFU/g to 2.5 x 10^2 CFU/g. In batch III with 648 AU/g of nisin the *Listeria* counts diminished immediately to 100 CFU/g and were reduced to 50 CFU/g after the 5th day. Thus, representing a reduction of 3.61 log CFU/g when compared to the control batch. The physicochemical characteristics of the product were evaluated by its pH, water activity, fat content and the ratio of NPN versus total nitrogen and N-a-amino versus total nitrogen. The product showed a water activity (aw) of 0.875, a final pH of 6.06, and a fat percentage of 41.50% (Table 1).

In cooked ham, an outgrowth of *L. innocua* CTC1014 was observed in the control batch (batch IV) during storage. *Listeria* grew from 5 MPN/g to 1.9 x 10^{10} CFU/g at the end of the 37 days of storage at 7ºC. When 1,600 AU/g of nisin were applied in batch V, the growth of *Listeria* reached 7.9 x 10^7 CFU/g. In batch VI with 4,800 AU/g of bacteriocins, the growth of Listeria at the end of the assay reached 2 x 10^2 CFU/g, thus representing a 7.98 log CFU/ g reduction when compared to the control batch. Physicochemical parameters of the product are in Table 1; a high aw (0.982) and a low percentage of fat (3.48%) were observed. In pate, an outgrowth of *L. innocua* CTC1014 was observed in the control batch (batch VII). *Listeria* grew up to 1.9 x 10^9 CFU/g at the end of the assay (37 days). The *Listeria* population in batch VIII with 1,600 AU/g of bacteriocin only grew to 1 x 10^2 CFU/g at the end of the process. Batch IX with 4,800 AU/g of nisin was able to keep *Listeria* under 3 MPN/g during the 37 days storage at 7ºC (Fig. 3). A high percentage of fat (20.01%) and a high ratio between NPN and total nitrogen content could be observed (23.97%) (Table 1). When minced meat pork was stored for 6 days at 7ºC, *L. innocua* CTC1014 grew from 5 MPN/g to 48 CFU/g in the control batch, but no growth was observed (<3 MPN/ g) when 1,600 AU/g

of nisin were applied (batch XI) (Fig. 4). The pH of the product was slightly acidic (5.9) and the percentatge of fat 13.09% (Table 1). In chicken breasts, *L. innocua* CTC1014 grew up to 6 3 105 CFU/cm2 in the control batch during the 7 days of storage at 7ºC (Fig. 5). The population of *Listeria* was reduced to 2.7 x 10^2 CFU/g when 3,200 AU/cm of nisin were applied (batch XV) and diminished to 3.6 MPN/ cm^2 when 4,800 AU/cm^2 of bacteriocin were applied (batch XVI), thus representing a 5.26 log reduction when compared to the control batch without added bacteriocin. The pH of the product was slightly acidic (5.78) and after 6 days storage of the product at 78C, the percentages of NMP/ Ntotal and the N-a-amino/Ntotal raised by a factor of 2.8 and 3.18, respectively (Table 1).

Initial inocula of *L. innocua* CTC1014 in the different products assayed were standardized about 10 CFU/g in order to reproduce the natural initial contamination of meat products as previously reported (7, 26). In espetec (slightly fermented sausage) the initial inoculum was established as 1 x 10^4 CFU/g because it had previously been reported that *Listeria* survives but not grow in fermented products (21, 22). Temperature abuse (7ºC) has been applied in order to simulate abusive storage conditions. In this study bacteriocin were applied as semipurified food additives as prior *in situ* assays demonstrated that ad dition of *Lactococcus lactis* ATCC 7962 in fermented sausages, hamburgers, and chicken breasts did not exert any positive antilisterial effect when compared to the control batch. It has also been reported that the growth rate of the producer strain and bacteriocin production was highly inhibited at refrigeration temperatures and by salt and pepper (essential ingredients for fermented sausages) (2). The growth of *Listeria* in chicken breasts was high when compared to the growth of *Listeria* in minced pork meat during the 6 to 7 days of storage at 7ºC. Listeria grew from less than 10 MPN/g up to 48 CFU/g in minced pork meat and up to 6 x 10^5 CFU/cm^2 in chicken breasts. Similar results were obtained by Hugas *et al.* (23). The increasing amino acid content observed in the chicken breasts during the 6 days storage when compared to minced pork meat (Table 1) could in part explain the higher growth of *Listeria* in chicken breasts (17). Indigenous competitive microflora have also been proposed as a factor to explain the differential in growth of *Listeria* in minced pork meat while compared to chicken breasts (17, 26, 27). Other reported parameters linked to the growth of *Listeria* such as the higher water activity of the chicken breast and the higher thiamine, fat, and salt content of the minced pork meat could not be directly related to the growth of *Listeria* in the different products assayed in this study (17, 20, 41). In the batches treated with nisin a smaller amount of bacteriocins was needed to keep the *Listeria* counts under 3 MPN/g in the minced pork meat than in chicken breasts. Minced pork was fatter and had better bacteriocin adsorption than whole chicken breasts. In cooked ham and pate, *Listeria* grew very well and reached higher counts in cooked ham than in pate. In pasteurized products, the absence of competitive microflora seems to be the most important factor facilitating the growth of secondary contaminants, as there is no competition with the indigenous microflora of the raw meat (24). Besides, both products had a high aw and a pH over 6.0 (6.3), factors that have been described as benefiting the growth of *Listeria* (17). The higher fat and amino acid content of the pate did not promote the growth of *Listeria* in this product when compared to cooked ham. Without competitive microflora and in favorable physicochemical conditions, the small amino acid content of the cooked ham was enough to support a high outgrowth of *Listeria*. High numbers of *Listeria* are usually found in pate, 10^4 to 10^5 CFU/g (32). The batches of pate treated with 4,800 AU/g of cacteriocin were able to keep the *Listeria* counts under 3 MPN/ g while the batches of cooked ham treated with the same amount of bacteriocin reached 2 x 10^2 CFU/g. The results obtained in the raw (minced pork meat and raw chicken breasts) and cooked meat products (pate and cooked ham) seem to confirm that fat is important for the in situ activity of bacteriocin but not for Listeria growth. In espetec, *Listeria* grew 1 log cycle during the first 4 days of processing and then decreased to the initials counts. Thus, the physicochemical characteristics of the product, a_w (0.83 to 0.86), pH (6.0), nitrate (150 ppm), and nitrite (150 ppm) together with NaCl concentration (2.2%) and pepper (2‰) were able to inhibit the growth without eliminating *Listeria*. In dry fermented sausages, Hugas *et al.* (21, 22) observed 1-log reduction of *L. innocua* CTC1014 counts in the uninoculated batches. The lower pH (5.05) of dry fermented sausages was probably the main factor explaining the reduction of *Listeria* counts when compared to espetec. Grau and Vanderlinde (20) observed that Listeria grew better at pH 6.0 or higher than at 5.5 or less. The espetec sausages treated with 648 AU/g of nisin obtained an extra reduction of 3 log cycles in *Listeria* counts when compared to the control batch. With 400 AU/g of sakacin, Hugas *et al.* (21) obtained a 2.64 log reduction in *L. innocua* counts

when compared to the initial counts. Thus, bacteriocins may be considered as an additional anti-*Listeria* hurdle that could act cooperatively with the other additives and the global drying process typical in sausage manufacture.

CONCLUSION

Thus, bacteriocins may be considered as an additional anti-*Listeria* hurdle that could act cooperatively with the other additives and the global drying process typical in sausage manufacture.

Conflict of interest:
The authors declare that they have no conflict of interest.

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