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ORIGINAL ARTICLE

Detection of *Campylobacter* spp. and *Yersina* spp. in chicken burgers: Critical evaluation of CIN medium and characterization of psychrotolerant microbiota in commercial samples from Reus (Tarragona, Spain)

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KEYWORDS
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Abstract Two of the three most prevalent bacterial genera associated with human diseases caused by meat consumption are *Campylobacter* (mainly in poultry meat) and *Yersinia* (in pork meat). As its detection and quantification is not regulated by the European legislation (except for the quantification of *Campylobacter* in poultry channels), several samples of chicken burgers from different establishments including supermarkets and retail trade outlets in Reus (Spain) were processed to ensure microbiological safety. Microbiological criteria and procedures have been those included in the European standards for *Campylobacter* (Regulation No. 2017/1495) and *Yersinia* (ISO 10273:2917). Results showed the absence of *Campylobacter* spp. in all samples, but high counts (10^4 to 5×10^5 CFU/g) of typical colonies (''red bull's eye'' morphology) on CIN medium compatible with *Yersinia* spp. The biochemical profile of the strains from the typical colonies, and their subsequent molecular identification using MALDI-TOF MS enabled us to identify *Yersinia intermedia* in just one sample, and *Pseudomonas* and *Serratia liquefaciens* in the remaining ones. These findings call into question the usefulness of CIN medium for detecting *Yersinia* spp. in food. Moreover, the presence of high counts of psychrotolerant bacteria from the genera *Pseudomonas* and *Serratia* highlight the need to improve hygienic conditions in the procedures used to produce meat derivatives.

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PALABRAS CLAVE
Control;
Enfermedades transmitidas por alimentos;
MALDI-TOF;
Seguridad

Detección de *Campylobacter* spp. y *Yersinia* spp. en hamburguesas de pollo: evaluación crítica del medio CIN y caracterización de microbiota psicotolerante en muestras comerciales adquiridas en Reus (Tarragona, España)

Resumen Dos de los tres géneros bacterianos más prevalentes en enfermedades humanas asociadas al consumo de productos cárnicos son *Campylobacter* (mayoritariamente en carne de ave) y *Yersinia* (en carne de cerdo). Dado que su detección y cuantificación no está regulada por la legislación europea (excepto la cuantificación de *Campylobacter* en canales de aves), para estudiar la ausencia de riesgo microbiológico se decidió procesar varias muestras de hamburguesas de pollo procedentes de diferentes establecimientos entre los que se encontraban supermercados y puntos de venta de comercio minorista de la ciudad de Reus (España). Los criterios y procedimientos microbiológicos han sido los contemplados en las normas europeas para *Campylobacter* (Reglamento 2017/1495) y la norma ISO 10273:2917 para *Yersinia*. Los resultados mostraron la ausencia de *Campylobacter* spp. en todas las muestras, pero recuentos elevados (10^4 a 5×10^5 UFC/g) de colonias típicas (morfología ''ojo de buey rojo'') en medio CIN compatibles con *Yersinia* spp. El perfil bioquímico de las cepas de las colonias típicas en CIN y su posterior identificación molecular mediante MALDI-TOF MS nos permitió identificar a *Yersinia intermedia* en una sola muestra, mientras que el resto de las colonias fueron identificadas como *Pseudomonas* spp. y *Serratia liquefaciens*. Debido a estos hallazgos, la utilidad del medio CIN para la detección de *Yersinia* spp. en alimentos queda en entredicho. Además, debe considerarse la necesidad de mejorar las condiciones higiénicas en los procedimientos utilizados para producir derivados cárnicos, debido al elevado recuento de bacterias psicrotolerantes pertenecientes a los géneros *Pseudomonas* y *Serratia*.

Introduction

Foodborne diseases (FBDs) are an important and growing public health concern worldwide[15]. According to the World Health Organization, these diseases may be caused by the consumption of food contaminated with microorganisms, such as bacteria, viruses or parasites or chemical substances like heavy metals[27]. The three types of foodborne illness are: intoxication (the toxin produced by the pathogens causes food poisoning), infection (caused by the ingestion of food containing pathogens), and toxic-infections (pathogens producing toxins while growing in the human intestines)[3,10,23]. Moreover, the Foodborne Disease Burden Epidemiology Reference Group (FERG) of the World Health Organization estimated that 31 FBDs caused over 600 million cases of illnesses and 420 000 deaths worldwide in 2010. Furthermore, the Food and Drug Administration (FDA) estimates there are about 48 million cases of foodborne illness each year in the U.S. population[19]. The contamination of food can be produced at any stage of the manufacturing, delivery and consumption chains, and consequently the consumption of contaminated food or water can result in a FBD in the host[1,12,16,17]. In addition, food animals are the major reservoirs of many food-borne zoonotic bacterial pathogens, and food products of animal origin are the main vehicles of transmission[1,11,14]. Bacteria can reach the gastrointestinal tract and proliferate, leading to the secretion of toxins and structural virulent factors that are responsible for their pathogenesis. This can produce a wide range of illnesses in the host, the most frequent being enteritis with or without moderate fever[10,15,27]. The principal bacterial pathogens that are responsible for most FBDs are species belonging to the genera *Campylobacter*, *Listeria*, *Salmonella*, *Shigella*, *Yersinia* and *Escherichia coli*[1,11,18.23]. Due to the high incidence of these microorganisms, public health surveillance includes their detection (and eventual quantification), establishing adequate control mechanisms over the foods involved as vehicles of poisoning, and elucidating the origin of epidemic outbreaks once they have occurred[11,27]. The main foods implicated in outbreaks of food poisoning are milk, eggs, fish, meat (usually from poultry and pork), and all their derivatives[1,16]. Specifically, poultry meat (mainly chicken) ranks first in terms of production on a global scale, and is the main route of transmission of some microorganisms causing poisoning in Europe, such as *Campylobacter* spp., and Enterobacteriales (i.e. *Salmonella*, *Shigella*, *Yersinia*, and *E. coli*)[1,2]. With respect to the control of *Campylobacter* spp., the Commission Regulation (EU) No. 2017/1495 establishes the microbiological criteria and hygienic procedures that must be fulfilled regarding *Campylobacter* in broiler carcasses. There is no official legislation for *Yersinia* spp. establishing maximum acceptable limits in food; however, ISO 10273:2917 is available to establish procedures for the detection of *Yersinia enterocolitica* in the food chain[20]. Nonetheless, the incidence of these infections is vastly underestimated, and since food poisoning is a major public health concern, a specific legislation to regulate maximum acceptable limits for *Campylobacter* spp. and *Yersinia* spp. in food is needed.

Table 1 Sources of chicken meat burger samples acquired in the city of Reus (Tarragona province, Spain). The term 'supermarkets' refers to large retail chains operating both in Catalonia and throughout Spain. The category 'stands' includes various individual stalls located in the Central Market of Reus.

Origin	Samples
Supermarket A	A1, A2, A3, A4
Supermarket B	B1, B2, B3, B4
Supermarket C	C1, C2, C3, C4
Supermarket D	D1, D2, D3, D4
Supermarket E	E1, E2, E3, E4
Stand F	F1, F2, F3, F4
Stand G	G1, G2, G3, G4
Stand H	H1, H2, H3, H4
Stand I	I1, I2, I3, I4
Stand J	J1, J2, J3, J4

The main objective of our study was to detect and quantify both bacterial genera in chicken meat burgers, and assess whether this food represents a potential risk to the health of consumers.

Material and methods

Sample collection

Reus, the capital city of the Baix Camp region, in the south of Tarragona province (Catalonia, Spain), was chosen as the site for sample collection, because its companies process around 200 000 chickens for meat/year (Nombre d'explotacions i capacitat per municipi. 2023) but also because the CESAC (Poultry Health Center of Catalonia and Aragon Communities) is located there. A total of 40 chicken burger samples were acquired, 20 from five national and international chains of supermarkets and the same number of samples from five commercial stands in the Central Market of Reus city (Tarragona province, Spain) (Table 1).

All burger samples were transported to the laboratory in a thermal bag at 4—7 °C and stored for 3 h in a refrigerator at 5 ± 1 °C before processing.

Sample processing

Samples were homogenized in a stomacher (Stomacher® 400 Circulator (Seward Ltd., West Sussex, UK)) in accordance with the UNE-EN ISO 10273:2017 standard and UE legislation No. 2017/1495. A mixture of 225 ml of sterile peptone water 0.1% (w/v) and 25 g of each sample was homogenized for 30 min at 230 rpm. Then, 1 ml of a homogenized mixture from each sample was aliquoted to tubes containing 9 ml of Ringer solution to prepare 1:10 and 1:100 dilutions.

Presumptive detection and quantification of *Campylobacter* spp. and *Yersinia* spp.

For presumptive detection and quantification of colony-forming-units (CFU) per gram of sample of *Campylobacter* spp. and *Yersinia* spp., 1 ml from direct samples and each dilution (1:10 and 1:100) was inoculated onto BD Campylobacter Agar (Butzler) for *Campylobacter* spp., using a Drigalski spatula, and the Petri dishes were incubated at 42 °C from 48 h to 7 days under microaerobic conditions (oxygen, 6–16%; carbon dioxide, 2–10%; and nitrogen, 80% using Gas Pak EZ Campy container sachets (Becton Dickinson, Sparks, MD, USA)). Petri dishes were checked every day for 7 days and colonies considered presumptive for *Campylobacter* spp. were those greyish/colorless, mucoid, with irregular borders and a tendency to spread through the inoculation streak. For detection and quantification of *Yersinia* spp. Yersinia Selective agar plates (CIN; Laboratories Conda S.A., Spain) with Yersinia Selective Supplement (CIN), in accordance with ISO 10273:2017, were inoculated. Petri dishes were incubated at 30 °C for 48 h, and the colonies with a red bull's eye appearance were considered presumptive for *Yersinia* spp.

Yersinia spp. phenotypic presumptive identification

For red bull's eye bacterial colonies from each burger sample, Gram staining, oxidase and urease tests, and growth on Kliger Iron Agar (KIA), Lysine Iron Agar (LIA), Sulfide Indole Motility (SIM), Motility Indole Ornithine (MIO), and Simmons Citrate Agar were performed[5]. In the absence of typical colonies in a processed burger sample, an atypical colony was phenotypically characterized.

MALDI-TOF MS identification

Only a set of strains representing the bacterial phenotypic diversity in the burger samples was selected for identification using this methodology. Identification was carried out using matrix-assisted laser desorption ionization time of flight (MALDI-TOF) coupled mass spectroscopy (MS), using MALDI Biotyper MSP Identification Standard Method 1.1 (Bruker®) version 9 of the Biotyper database. MALDI-TOF MS identifications were classified using modified score values proposed by the manufacturer: a score value $\geq$2 indicated species identification; a score value between 1.7 and 1.9 indicated genus identification, and a score value <1.7 indicated no identification.

Statistical analysis

To compare the means of two independent groups (red bull's eye CFU/g vs. atypical CFU/g), a two-sample *t*-test was used if the data were normally distributed; otherwise, the Mann–Whitney *U* test was performed for non-normal distributions. Shapiro–Wilk test was used to evaluate if the variables were or were not normally distributed. Based on this result, either the *t*-test or the Mann–Whitney *U* test were performed. Statistical analyses were conducted using Julius (https://julius.ai/chat).

Results

Presumptive detection and quantification of *Campylobacter* spp. and *Yersinia* spp., and statistical analysis of the results

Campylobacter spp. presumptive colonies were not detected in any of the processed samples and dilutions. Consequently, we assumed that *Campylobacter* spp. were either absent in the analyzed samples, or present at concentrations below 10 CFU/g food, which corresponds to the lower limit of detection of the methodology used.

On CIN medium, two types of colonies, differing in size and color, were observed. On the one hand, small colonies with a diameter of 0.5−1 mm were detected after 24 h of incubation at 30 °C, displaying an intense pink color at the center surrounded by a transparent or translucent halo, characteristic of the ''red bull's eye'' appearance and morphologically compatible with most strains of *Y. enterocolitica*. On the other hand, larger colonies (with a diameter greater than 2 mm), intensely fuchsia in color without a surrounding halo, either colorless or slightly colored, were also observed. They were considered ''atypical colonies'', as they usually correspond to taxa other than *Yersinia* spp., although other strains of the genus may present this phenotype. The differential counts of the colonies grown on CIN are shown in Table 2. The comparison between red bull's eye and atypical CFU/g counts for each sample is presented in Figure 1. There is clearly a higher median and more variation in the atypical CFU/g measurements. The heat map of typical and atypical colonies on CIN (Fig. 2) indicates that the highest concentrations of both colony types are in the lower-central section of the sampling grid, corresponding to samples collected from the commercial stands at the Central Market of Reus. Notably, red bull's eye CFU/g exhibits greater variability, with numerous samples showing no growth, whereas atypical colonies generally display higher counts and a more consistent presence across the sampled locations. Based on the Shapiro–Wilk test results, both variables are not normally distributed (p-value <0.05). Given this non-normality, the non-parametric Mann–Whitney U test was used instead of a t-test. Mann–Whitney U test results were: statistic = 404.0; p-value = 0.000132. The Mann–Whitney U test shows a highly significant difference between the two groups ($p < 0.001$), confirming a non-parametric approach. The Spearman Rank Correlation coefficient was 0.385237, and the p-value: 0.0141. There is a moderate positive correlation ($\rho \approx 0.39$) between red bull's eye and atypical CFU/g, which is statistically significant ($p < 0.05$). The Median test result was 12.808005, with a p-value of 0.000345. The Median test confirms significant differences between the groups ($p < 0.001$), indicating that the central tendencies of the two groups are different. Moreover, the Cliff's Delta (effect size) was approximately −0.50, indicating a medium to large effect size, suggesting that atypical CFU/g tends to have higher values than red bull's eye CFU/g. Statistics are shown in Table 3. In brief: the atypical CFU/g has a higher mean (303 925) compared to red bull's eye CFU/g (79 400); both measurements show considerable variation; and there are samples with 0 CFU/g in both categories.

Table 2 Enumeration of typical (red bull's eye; presumptive *Yersinia* spp.) and atypical colonies count on CIN medium. The data show significant variation, and several samples presented no detectable CFU/g in either category.

Sample	Typical colonies (CFU/g[a])	Atypical colonies (CFU/g)
A1	3×10^4	6×10^4
A2	3×10^4	3.6×10^5
A3	NG[b]	4×10^4
A4	5×10^4	4×10^5
B1	1×10^4	9×10^4
B2	NG	NG
B3	NG	1×10^5
B4	1×10^4	1.8×10^5
C1	NG	3.1×10^5
C2	NG	7.5×10^5
C3	NG	8.3×10^5
C4	NG	6.3×10^5
D1	NG	7×10^4
D2	2×10^4	7×10^4
D3	1×10^4	6×10^4
D4	NG	9×10^4
E1	1×10^4	1×10^4
E2	1×10^4	NG
E3	1×10^4	1×10^4
E4	4×10^4	NG
F1	4.9×10^5	2.4×10^5
F2	8×10^4	1×10^4
F3	6.3×10^5	1.3×10^5
F4	2.4×10^5	1.4×10^5
G1	1.5×10^5	8.9×10^5
G2	1.4×10^5	5.2×10^5
G3	2×10^4	5.9×10^5
G4	9×10^4	6.3×10^5
H1	2×10^4	1.5×10^5
H2	1×10^4	1.5×10^5
H3	5×10^4	1.9×10^5
H4	2×10^4	2.9×10^5
I1	4×10^5	UNC[c]
I2	1.7×10^5	UNC
I3	2.5×10^5	UNC
I4	1.4×10^5	UNC
J1	1.6×10^4	8.4×10^4
J2	1×10^4	6×10^4
J3	2×10^4	1.8×10^4
J4	NG	NG

[a] Colony forming units per gram of sample.
[b] No growth.
[c] Uncountable.

Phenotypic presumptive identification

Gram negative bacterial strains from A1 to D4, E3 and I4 samples had a biochemical profile compatible with *Pseudomonas* spp. (family Pseudomonadaceae, class Gammaproteobacteria) (Supplementary table; grey background). Bacterial strains from E1, E2, E4, G2, H1 and J2 samples showed a biochemical profile compatible with *Serratia liquefaciens* (family Enterobacteriaceae, class Gammaproteobacteria) (Table 2; salmon background); however, the bacterial strain

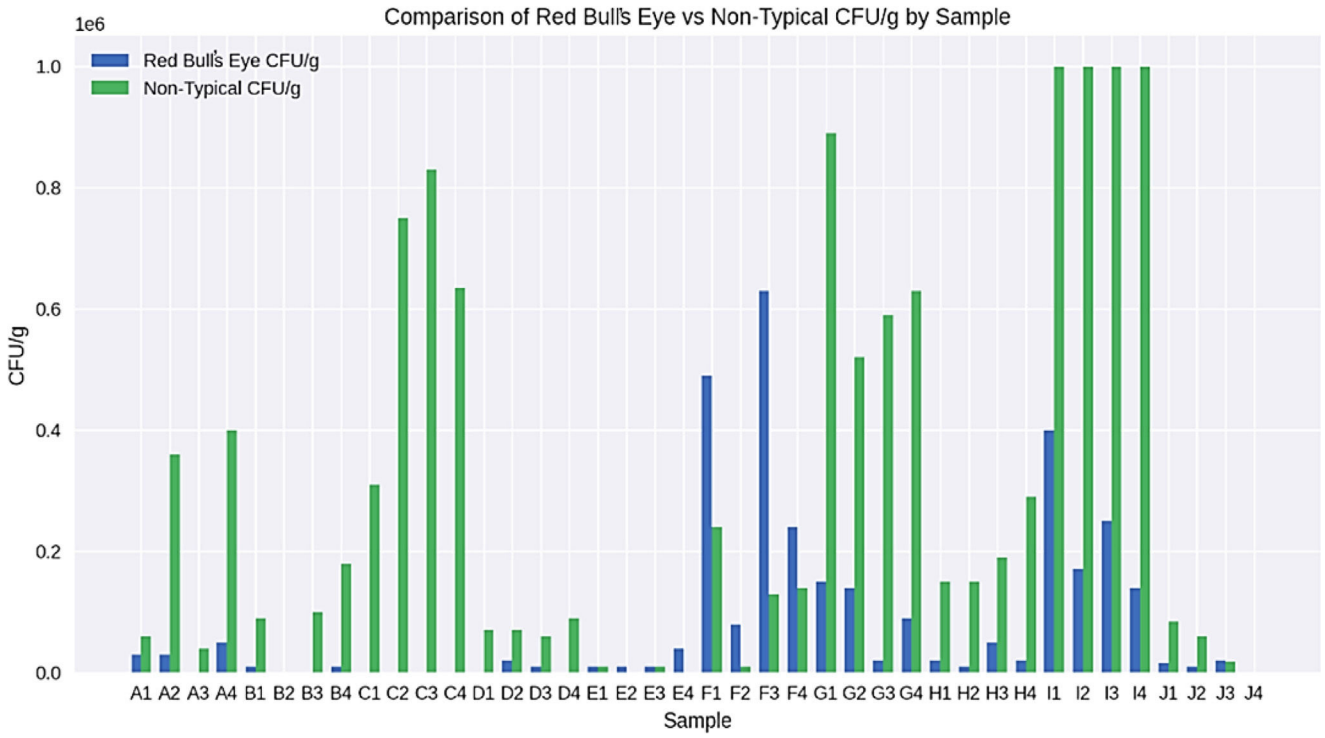

Figure 1 Bar chart shows comparing red bull's eye and atypical counts as CFU/g (colony forming units per gram of sample processed). The data reveal a noticeably higher median and increased variability among the atypical colony counts.

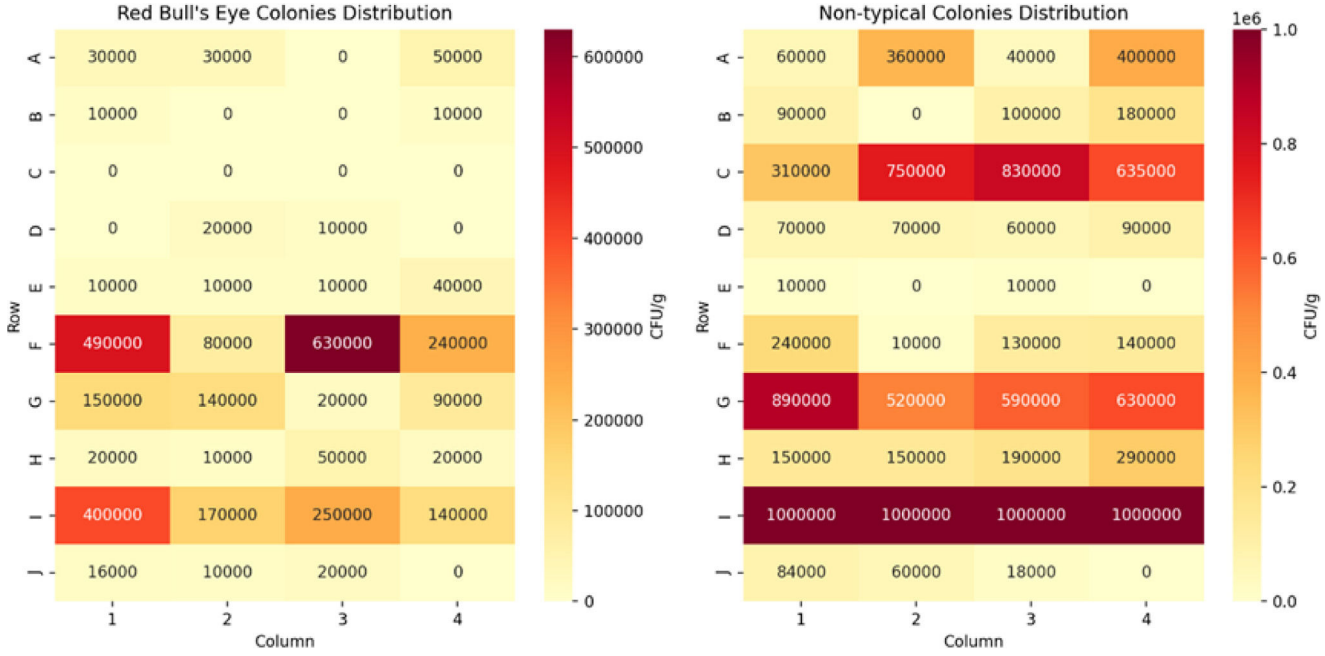

Figure 2 Heat map showing the distribution of typical and atypical colony counts (CFU/g) on CIN medium by sample. The highest counts for both colony types are concentrated in the lower-central section of the sampling grid, corresponding to samples obtained from commercial stands at the Central Market of Reus. Red bull's eye (typical) colonies exhibit greater variability, with several samples showing no detectable growth, whereas atypical colonies tend to present higher counts and a more consistent presence across the sampling area.

Table 3 Summary statistics of typical (red bull's eye; compatible with *Yersinia* spp.) vs. atypical colony counts. The atypical colonies have a higher mean (303 925) compared to red bull's eye colonies (79 400) count.

Parameter	Typical colonies (CFU/g)	Atypical colonies (CFU/g)
Count	40	40
Mean	79400	303925
Std[a]	141555.6065424106	339712.8793328498
Min[b]	0	0
25%	7500	60000
50%	20000	145000
75%	82500	537500
Max[c]	630000	1000000

[a] Standard deviation.
[b] Minimum value.
[c] Maximum value.

Table 4 MALDI-TOF MS-based identification of bacterial strains with red bull's eye morphology on CIN.

Strain	MALDI-TOF score value	Identification
A1	2.066	*Pseudomonas libanensis*
A3	2.026	*Pseudomonas tolaasii*
B2	2.097	*Pseudomonas libanensis*
C2	2.117	*Pseudomonas libanensis*
C3	2.246	*Pseudomonas libanensis*
D2	2.147	*Pseudomonas synxantha*
D4	2.183	*Pseudomonas libanensis*
E1	2.438	*Serratia liquefaciens*
E2	2.387	*Serratia liquefaciens*
G2	2.452	*Serratia liquefaciens*
H1	1.801	*Serratia* sp.
H4	2.424	*Yersinia intermedia*
I4	1.982	*Pseudomonas* sp.
J2	2.242	*Serratia liquefaciens*

from H4 showed a profile compatible with *Yersinia* sp. (Supplementary table; blue background). The remaining bacterial strains belonged to the family Enterobacteriaceae (class Gammaproteobacteria) (Supplementary table; white background).

MALDI-TOF identification

Using MALDI-TOF the bacterial identity at the species level was confirmed when the score value was >2, the spectrum quality was classified as ++/+++, and the consistency index was A or B. Overall, general, our results showed higher scores than 2.0 and *Yersinia intermedia* was identified only in one case. The rest of the bacterial strains corresponded to species of the genus *Pseudomonas* and *Serratia* (Table 4).

Discussion

The WHO published a report estimating 600 million cases of foodborne illness and 420 000 deaths in 2010. Children less than 5 years-old is a high susceptible population, with an estimated rate of 120 000 deaths per year attributed to unsafe food. This report[28] examined the global public health burden of infections based on 31 foodborne hazards. These data highlight the importance of implementing food safety measures to protect vulnerable populations.

FBDs represent one of the most common and important public health issues worldwide. According to WHO, 23 million people in the European Union (EU) fall ill, and 5000 die every year due to FBDs[29]. The Centers for Disease Control and Prevention estimate that FBDs affect 48 million people annually, with 128 000 hospitalizations and 3000 deaths in USA[10]. *Campylobacter* is the most common genus involved in human bacterial gastroenteritis globally, and campylobacteriosis is commonly reported as zoonosis in the EU since 2005[26].

Microbiological guidelines, such as Hazard Analysis and Critical Control Points, Good Manufacturing Practice, and good hygienic practices developed by the WHO and the United States Food and Drug Administration should be implemented strictly to prevent *Staphylococcus aureus* contamination. Areas where MRSA patients are nursed should be thoroughly cleaned using disinfectants[7].

Molecular methods, including mass spectrometry and PCR-based multiplex panels have been developed for the detection of enteric bacteria, and some laboratories are beginning to incorporate the techniques[9]. These methods have the potential to improve our ability to provide rapid and accurate identification, but it is also important to understand their limitations. Commercial MALDI-TOF MS instruments can provide rapid identifications of *Salmonella* and *Yersinia*, but a limited identification of *Shigella* spp. In our study we used MALDI-TOF for strain identification, which increased the veracity of the results[8].

The parametric tests consistently show significant differences between the two groups of bacterial colonies, red bull's eye and atypical, growing on CIN. There is a moderate positive correlation between the two measurements, such as CFU/g. The effect size is substantial, indicating meaningful practical differences. The atypical CFU/g consistently shows higher values than red bull's eye CFU/g. Based on our results, high counts of *Pseudomonas* spp. and of *S. liquefaciens* in chicken burger samples acquired in supermarkets, but specially in the stands of the Central Market of Reus. *Pseudomonas* spp. and of *S. liquefaciens* colonies growing on cefsulodin–irgasan–novobiocin (CIN) agar resemble those of *Yersinia* spp. (red bull's eye aspect), which is a great inconvenience for a differential count and to isolate *Yersinia* spp. to improve their identification. Although Schiemann, the developer of the CIN medium[24], reported that it inhibits the growth of *Pseudomonas aeruginosa* and *Serratia marcescens*, other members of the Enterobacteriaceae family – such as *Citrobacter freundii* and *S. liquefaciens* – are capable of growing on the medium and may produce colonies that resemble those of *Yersinia* species[25]. Despite the absence of *Campylobacter* spp. and *Yersinia* spp. (except for one strain of *Yersinia intermedia*) in our samples, the high levels of campilobacteriosis[13] and yersiniosis in Spain[21] underscore the importance of continuing to study its presence in foods of animal origin.

Food contamination by *Pseudomonas* spp. mainly occurs through contact with contaminated water, floor particles, and inefficient surface decontamination that come into

contact with food[6]. The genus *Pseudomonas* is the most frequently implicated in food spoilage because it can produce biofilms[6] and extracellular enzymes, such as various proteases and lipases, which are often heat-resistant, leading to spoilage and stability problems in food[4,22]. Additionally, *Pseudomonas* spp. are usually isolated from food along with other genera, such as *Listeria*, *Salmonella* or *Serratia*.

Conclusion

The presence and high counts of *Pseudomonas* spp. and *S. liquefaciens* in poultry meat burgers could be attributed to inadequate washing of the meat, insufficient disinfection of food processing surfaces, or excessively long refrigeration periods aimed at maximizing poultry shelf life. Consequently, we highlight the need to improve hygienic and sanitary conditions in meat derivative processes, considering the high levels of contamination by psychrotrophic Gram-negative bacteria. Moreover, our study raises questions about the high selectivity of CIN medium for detecting *Yersinia* spp. in food. In light of these findings, more selective culture media should be developed to detect *Yersinia* spp. in food samples.

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Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version available at https://doi.org/10.1016/j.ram.2025.06.002.

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