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Yersinia enterocolitica detection in pork products: Evaluation of isolation protocols

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ABSTRACT

Conventional methods for *Yersinia enterocolitica* detection in food samples are generally considered inadequate. Problems arise from the presence of the so-called "background flora", coupled to the low contamination level of the pathogen. Since, data on the microbial ecology occurring in competitive microflora are still lacking, MALDI TOF MS was used for strains 'identification after enrichment in PSB or ITC broths, and after plating on selective CIN medium at different incubation times. SYBR Green Real time PCR was used for the *Y. enterocolitica* strains' detection (4/O:3, 1A/O:5) in experimentally contaminated foods, as well as in naturally contaminated samples. A higher number of different bacterial genera (10 on CIN and 18 on PCA) was recorded after enrichment in PSB, whilst enrichment in ITC led to recovery of 6 and 10 genera on CIN and PCA, respectively. Yersiniaceae was the dominant family on the first day of incubation, but on the second day the percentage of isolation considerably decreased. By testing experimentally contaminated samples, substantial difficulties were encountered. The biotype 1A was always detected, whereas strain 4/O:3 proved to be poorly competitive. Based on the data, the enrichment media PSB and ITC, currently proposed for *Y. enterocolitica* detection, need to be improved to promote a successful pathogen's recovery.

1. Introduction

Yersiniosis, mainly caused by the species *Yersinia* (*Y.*) *enterocolitica,* is the fourth most commonly reported bacterial food-borne zoonosis in the European Union (EFSA European Food Safety Authority, 2019). *Y. enterocolitica* is a facultative anaerobic, Gram-negative coccoid rod belonging to the family of Yersiniaceae. *Y. enterocolitica* is divided into six biotype (1A, 1B, 2, 3, 4, 5) all pathogenic for humans, except biotype 1A (Bancerz-kisiel, 2018). The biotype 1A has always been regarded as avirulent because it does not contain the major chromosomal virulence gene (*ail*, *ystA*) and does not harbour the virulence plasmid pYV (Paixão et al., 2013). However, since this biotype has been isolated from stools of diarrheic patients, it is nowadays considered as potential pathogenic (Tuompo et al., 2017). *Y. enterocolitica* can further be dived in more than 70 serotypes, with bio-serotype 4/0:3 as most commonly associated with human infections (Torres et al., 2018).

Pigs are considered the main reservoirs of *Y. enterocolitica,* and pork and pork products have been identified as major sources for human infection (Petsios et al., 2016). However, the pathogen's incidence is probably underestimated as the isolation and identification is complicated by the presence of a rich and varied accompanying flora on the agar isolation plates, combined with a low level of *Y. enterocolitica* in the samples, often resulting in false negative results (Petsios et al., 2016). Although molecular techniques based on species-specific genomic targets may improve the detection rate of *Y. enterocolitica* in foods, a subsequent isolation step to characterize the isolate remains necessary. The reference method, ISO 10273, for *Y. enterocolitica* isolation involves an enrichment in selective broths [e.g. peptone sorbitol bile (PSB) and irgasan-ticarcillin-potassium chlorate (ITC)], and isolation on selective agar media, followed by biochemical tests for the species confirmation

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(Zadernowska et al., 2014). According to the International Organization for Standardization, Cefsulodin-Irgasan-Novobiocin (CIN) agar is the preferred medium for *Y. enterocolitica* isolation, since typical colonies appear as characteristic dark red "bull's eyes" with a translucent edge (Petsios et al., 2016). However, though this isolation agar contains selective antimicrobials that inhibit the growth of other bacteria, some microorganisms escape this suppression and occasionally even exhibit the typical *Y. enterocolitica* colony's morphology (Morka et al., 2018).

Currently, knowledge on the outgrowth of non-*Yersinia* microorganisms in the enrichment broths, and on CIN agar, as their impact on the recovery of *Y. enterocolitica* strains is lacking. Moreover, conventional biochemical tests for identification and confirmation of *Yersinia* spp. are time-consuming (Peruzy et al., 2019b). Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) has emerged as a rapid and accurate method for routine identification for different clinical and foodborne relevant microorganisms (Cherkaoui et al., 2010; Peruzy et al., 2019a; Yu et al., 2019).

The aims of the present work were: i) to evaluate *Y. enterocolitica* strains (4/O:3 and 1A/O:5) detection in experimentally contaminated food samples through SYBR Green real time PCR, ii) to study the occurrence of *Y. enterocolitica* on pork carcasses and in several pork meat products collected at retail level, and iii) to assess the non-Yersinia microbial community on CIN agar plates as well as on Total Plate Count Agar (PCA) after two different selective *Yersinia* enrichments using MALDI TOF MS analysis.

2. Materials and methods

2.1. Sampling

Pork products examined were slices, minced meat, salami and fresh sausages. All samples of around 200–300 g were purchased at retail level in the Campania region (Southern Italy). Furthermore, pork carcasses were sampled directly after slaughter, but before chilling. For each half of a carcass, four areas (10 × 10 cm each) were swabbed using cellulose sponges (WPB01245WA, Sigma-Aldrich) rehydrated with 10 mL of sterilized Peptone Water (PW; CM0009, Oxoid, Basingstoke, UK). The surface sites sampled were: ham, back (loin-near the split surface), jowl and belly (near the split surface). Sampling was carried out according to indications provided by the EU Regulation 2073/2005. Subsequently, the four swabs per carcass were pooled and processed as one sample. All specimens were kept at 4 °C until analysis.

2.2. Detection of Y. enterocolitica in experimentally contaminated pork slices by SYBR Green PCR

To point out the optimal day for *Yersinia*'s recovering throughout enrichment, pork slices were experimentally contaminated with a *Y. enterocolitica* biotype 1A serotype O:5 (1A/O:5) and with a pathogenic *Y. enterocolitica* biotype 4 serotype O:3 (4/O:3) strain. Both strains were obtained from the Pathogenic Enterobacteria Collection of the Istituto Superiore di Sanità (Rome, Italy). Strains were cultured in Tryptone Soy Broth (TSB; CM0129, Oxoid, Basingstoke, UK) and incubated at 30 °C for 24 h, before seeding the pork slices by dipping at low (10^2–10^3 CFU/g – LC), medium (10^2–10^3 CFU/g – MC) and high (10^4–10^5 CFU/g – HC) level. The non-enteropathogenic *Y. enterocolitica* 1A strain was inoculated at low and medium concentrations only. Contamination levels were determined by prior counting on Plate Count Agar (PCA; CM0325, Oxoid). In detail, 25 g of pork samples were added of 225 mL (1:10 wt/wt) of sterilized Peptone-Sorbitol-Bile broth (PSB; 4022702, Biolife, Milan Italy), transferred in a sterile stomacher bag and treated for 3 min at 230 rpm using a peristaltic homogenizer (BagMixer®400 P, Interscience, Saint Nom, France). Homogenates were then seeded with *Y. enterocolitica* strains at the above-indicated contamination levels. After inoculation, samples were handly shaken for one min to optimize the distribution of the inoculum. Samples seeded with biotype 1A were incubated at 25 °C. Samples seeded with the biotype 4/O:3 were incubated in parallel at 25 and 10 °C for 8 days. In order to evaluate the recovery of *Y. enterocolitica*, DNA was extracted from the PSB broths at time zero (d-0), and day 1, 2, 3, 4, 5, 6, 7 and 8 by using the Chelex-100-resin method (1422822, Biorad, Hercules, CA, USA). In detail, two mL of each homogenate were transferred into a 2 mL tube, and centrifuged for 10 min at 10,000×*g* at 4 °C. The supernatant was discarded, the pellet re-suspended in 300 μL of 6% Chelex 100 by vortexing, and incubated at 56 °C for 20 min and for further 8 min at 100 °C. The suspension was immediately chilled on ice for 1 min, and centrifuged for 5 min at 10,000 *g* at 4 °C. The gene *ystA* was used as a target for the detection of *Y. enterocolitica* 4/O:3 (Peruzy et al., 2017). Three μL of DNA were added to 22 μL of PCR mastermix containing 12.5 μL of Qiagen QuantiTect SYBR Green PCR Kit (1x), 0.025 μL of forward *ystA*-F (5′-ATCGACACCAATAACCGCTGAG-3′), 0.025 μL reverse primers *ystA*-R (5′-CCAATCACTACTGACTTCGGCT-3′) and 9.45 μL of H_2O. The presence of strain biotype 1A was assessed by using *ystB* gene as target (Peruzy et al., 2017). Three μL of DNA were added to 22 μL of PCR mix containing: 12.5 μL of Qiagen QuantiTect SYBR Green PCR Kit (1x), 0.0375 μL of forward *ystB*-F (5′-GTACATTAGGCCAAGAGACG-3′), 0.0375 μL reverse primers *yst*B-R (5′-GCAACATACCTCACAACACC-3′) and 9.425 μL of H_2O. The fluorescence of SYBR Green and the melting curve were generated by using the CFX96 system (Bio-Rad). A specific melting temperature (Tm) of 78.5 ± 1 °C indicated a positive result for both targets. Two mL of PSB samples' homogenates were collected prior to *Y. enterocolitica* strain's inoculum and checked for the pathogen's absence by SYBR Green PCR as well.

2.3. Detection of Y. enterocolitica in naturally contaminated meat samples by SYBR Green PCR

Samples of minced meat (n = 7), slices (n = 7), salami (n = 7), sausages (n = 7) and seven samples from pork carcasses were analysed in order to detect *Y. enterocolitica*. Ten grams of samples or ten mL of sponge eluates were homogenized in PSB broth (90 mL) for 3 min at 10,000×*g* and incubated at 25 °C up to 5 days. DNA extraction from the PSB broths was performed using the Chelex-100-resin method as described above after one, two and five days of incubation. SYBR Green PCR was carried out as described in detail in Sect. 2.2.

2.4. MALDI-TOF MS analysis of cultures isolated from Y. enterocolitica isolation media

Samples of slices (n = 2) and minced pork meat (n = 2) and samples of two pork carcass halves were analysed for the presence of *Y. enterocolitica*. Ten grams of samples or ten mL of sponge eluates were homogenized in PSB broth (90 mL) as described above. Subsequently, 10 mL of each homogenate was transferred into 90 mL of sterile Irgasan–Ticarcillin–Potassium chlorate (ITC; 1361, CONDA, Torrejón de Ardoz, Spain) broth with ITC Supplement (6051, CONDA, Torrejón de Ardoz, Spain). Both ITC and PSB broths were incubated at 25 °C for 2 and 5 days, respectively. For the detection of *Y. enterocolitica* by SYBR Green PCR, DNA was extracted from the PSB broths at day 1, 2 and 5, as well as from ITC broths at the 2nd day. The procedure followed was the same as described in 2.2 section.

Moreover, at days 1, 2 and 5, PSB enrichments of the six different pork samples were submitted to ten-fold serial dilutions in Buffered Peptone Water (BPW; CM0509, Oxoid) followed by spread-plating on Cefsulodin-Irgasan-Novobiocin (CIN; CM0653, Oxoid) agar with *Yersinia* Selective Supplement (SR0109, Oxoid), as well as on PCA. ITC enrichments as indicated by the ISO methods (ISO: 10273:2017) were plated on CIN and PCA at day 2 (d-2) only. From the plates elected for counting, 20 colonies were randomly picked, and subcultured on PCA by the streak-plate method.

A total of 960 isolates from CIN (n = 480) and PCA (n = 480) plates were identified using MALDI-TOF MS. For the "Ethanol/Formic Acid

extraction" procedure from Bruker Daltonics, individual colonies were picked and suspended in 800 μL of TSB and incubated at 28 °C for 24 h. Subsequently, cultures were centrifuged (1533×*g* at 4 °C, 10 min), the supernatant was discarded and the pellet was washed twice with 500 μL of Milli-Q water. After centrifugation, the supernatant was discarded and the pellet resuspended in 100 μL of Milli-Q water with 50 μL of formic acid and 50 μL of acetonitrile. After mixing by pipetting followed by centrifugation (1533×*g* at 4 °C, 10 min), one μL of the supernatant was spotted onto a 96-spot plate and allowed to dry at room temperature. Afterward, the sample was covered with one μL matrix solution containing 10 mg/mL α-cyano-4-hydroxycinnamic acid in acetonitrile, deionized water, and trifluoracetic acid (50:47.5:2.5, [vol/vol/vol]). Samples were processed in the Microflex™ LT MALDI-TOF mass spectrometer (Bruker Daltonics) equipped with a nitrogen laser (l1/4337 nm) operating in linear positive ion detection mode using MALDI Biotyper Automation Control 2.0 (Bruker Daltonics). Identifications were obtained by comparing the mass spectra to the Bruker MSP database (version DB5989) using the Bruker Compass software (Bruker Daltonics) at default settings. Identification score criteria were classified according to the manufacturer's identification criteria; scores between 2.0 and 3.0 indicated highly probable species identification, between 1.7 and 1.99 certain genus and probable species identification, whereas values lower than 1.7 indicated a non-reliable identification. The analysis was repeated when the spots resulted in 'no peaks found'.

2.5. Statistical analysis

To compare the bacterial counts, one-way analysis of variance (ANOVA) was calculated by PAST software package (https://folk.uio.no/ohammer/past/). A probability value of less than 0.05 ($p < 0.05$) was defined as statistically significant.

3. Results

3.1. Recovery of Y. enterocolitica in experimentally contaminated samples

The presence of the *ystB* and *ystA* genes in the two *Y. enterocolitica* strains used in the present work was confirmed prior to the inoculation in the meat samples.

Strain biotype 1A, with gene *ystB* as target, was always detected in the samples inoculated at low and medium concentration during the entire period (Table 1). Strain biotype 4/O:3 with *ystA* gene as target was never detected in samples at lowest level (10–10^2 CFU/g), independently of the incubation temperature (Table 1). On the other hand, in pork slices contaminated with the intermediate pathogen level, *Y. enterocolitica* 4/O:3 was detected from the first up to the 3rd day in PSB broth incubated at 25 °C, and by from the day five up to day 8 when incubation was carried on at 10 °C (Table 1).

When slices were seeded at the highest level (10^4–10^5 CFU/g), strain 4/O:3 was detected from day 1 till 7, and from day 2 till 8 in samples incubated at 25 and 10 °C, respectively (Table 1).

3.2. Y. enterocolitica detection by SYBR Green PCR in pork products

In the 35 samples analysed, the *ystA* gene was never detected, while *ystB* gene was amplified in 13 out of 35 (37.14%) (Table 2). In detail, the *ystB* gene was detected in one carcass sample, two pork slices, four fresh sausages and six minced meat samples (Table 2). Moreover, in minced meat, the *ystB* gene was detected in ITC broth at day 2 as well. In eight samples (4 salami, 3 minced meat, 1 meat slices out of 7 samples always), the *ystB* gene was retrieved throughout the incubation period, while in one carcass sample, it was detected only from the day two. In one minced meat sample, the gene was detected only at the 1st and the 2nd day of incubation, whereas in two minced meat samples and one pork slices just at the 2nd day of incubation (Table 2).

Table 1
Detection of *ystB* and *ystA* genes, in artificially contaminated pork slices, by SYBR Green PCR as target for *Yersinia enterocolitica* strains 1A and 4/O:3, respectively. Melting temperature of 78.5 ± 1 °C indicated a positive result (D.) DNA extracted from PSB broth.

Inoculum level	Days d-0	d-1	d-2	d-3	d-4	d-5	d-6	d-7	d-8
	Yersinia enterocolitica 1A presence (D.) by *ystB* gene detection – incubation at 25 °C								
LC[a]	n.D.[b]	**D.**	**D.**	**D.**	**D.**	**D.**	**D.**	**D.**	**D.**
MC	n.D.	**D.**	**D.**	**D.**	**D.**	**D.**	**D.**	**D.**	**D.**
	Yersinia enterocolitica 4/O:3 presence (D.) by *ystA* gene detection - incubation at 25 °C								
LC	n.D.	n.D	n.D	n.D	n. D.	n. D.	n. D.	n. D.	n. D.
MC	n.D.	**D.**	**D.**	**D.**	n. D.	n. D.	n. D.	n. D.	n. D.
HC	n.D.	**D.**	**D.**	**D.**	**D.**	**D.**	**D.**	**D.**	n. D.
	Yersinia enterocolitica 4/O:3 presence (D.) by *ystA* gene detection - incubation at 10 °C								
LC	n.D.	n. D.	n. D.	n. D.	n. D.	n. D.	n. D.	n. D.	n. D.
MC	n.D.	n. D.	n. D.	n. D.	n. D.	**D.**	**D.**	**D.**	**D.**
HC	n.D.	n. D.	**D.**	**D.**	**D.**	**D.**	**D.**	**D.**	**D.**

[a] LC: Low (10^2–10^3 CFU/g – LC); MC medium (10^2–10^3 CFU/g – MC) and HC high (10^4–10^5 CFU/g – HC) pathogen's inoculum level.
[b] n.D: Not detectable.

Table 2
Detection of gene *ystB* by SYBR Green PCR used as target for *Y. enterocolitica* biotype 1A in 35 samples at the 1st, 2nd and 5th day of incubation at 25 °C in PSB broth.

Pork meat products	N. of samples		Days d-1	d-2	d-5
Salami	7	7	n.D.	n.D.	n.D.
Sausages	7	3	n.D.	n.D.	n.D.
		4	**D.**	**D.**	**D.**
Minced meat	7	1	n.D.	n.D.	n.D.
		2	n.D.	**D.**	n.D.
		1	**D.**	**D.**	n.D.
		3	**D.**	**D.**	**D.**
Meat slices	7	5	n.D.	n.D.	n.D.
		1	n.D.	**D.**	n.D.
		1	**D.**	**D.**	**D.**
Carcasses	7	6	n.D.	n.D.	n.D.
		1	n.D.	**D.**	**D.**

3.3. Evaluation of the accompanying bacterial flora occurring in enrichment and selective agar media

Enrichment for the six pork samples were carried out in PSB as well as in ITC broth. Both enrichment broths were then inoculated onto the selective CIN and PCA agar plates. The results of the bacterial counts on each plate are shown in Fig. 1. The viable counts on the CIN and PCA agar did not show significant differences ($p > 0.05$), and were in all cases higher than 8 Log CFU/g or cm^2 on the 1st day of enrichment in PSB, and on the 2nd day in ITC, independently by the analysed sample. From each CIN and PCA plate, 20, with a total of 960 colonies were randomly selected, and further analysed by MALDI-TOF MS. In general, regardless of the day of incubation, bacterial isolates on agar plates after incubation on PSB could be assigned to five families, 10 genera and 22 species on CIN and to nine families, 18 genera and 40 species on PCA. Bacterial isolates on CIN and PCA after incubation on ITC (day 2) could be

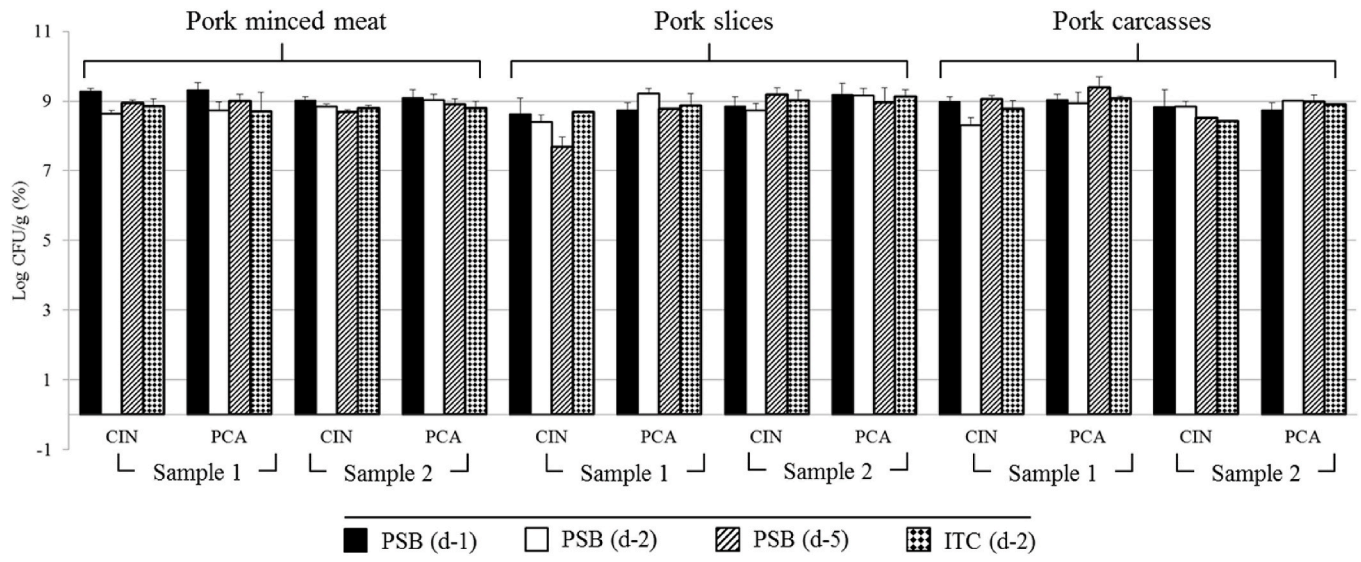

Fig. 1. Total bacterial counts on CIN and PCA agar plates of meat pork samples (2 carcasses, 2 minced meat and 2 slices) after enrichment in PSB or ITC.

assigned to 4 families, 6 genera and 11 species and to 6 families, 10 genera and 20 species, respectively. In meat slices, at the 1st day of incubation, members of Yersiniaceae family were the most frequently identified on both CIN (96, 80.8%) and PCA agar (68, 65.8%), respectively, but the percentages of isolation dropped at the 2nd, and even more at the 5th day of incubation for both media (Fig. 2). The same trend could be recorded for the minced meat as well as for the carcass samples. At day 2, enrichment in PSB provided a family distribution roughly overlapping with that obtained after enrichment in ITC (Fig. 2).

The distribution of the species is reported in Table 3a,b,c. After the 1st day of incubation, *Serratia* sp. was the dominant genus with 92 collected isolates on CIN (76.7%) and 75 on PCA (62.5%). At the 2nd day of incubation in PSB, *Citrobacter* sp. (n.50, 41.7%) along with *Serratia* sp. (n. 49, 40.8%) were the most commonly genera present on CIN medium, while *Proteus* sp. was the dominant one (n. 45, 37.5%) on PCA (Table 3b,c). After enrichment in ITC, the bacterial community was mainly composed of *Serratia* (n = 64, 53.8%) and *Morganella* sp. (n = 40, 33.3%) on CIN, and *Morganella* (n = 30, 25%), *Pseudomonas* (n = 27, 22.5%) and *Serratia* sp. (n. 26, 21.7%) on PCA. At the 5th day of incubation, almost 60% of the bacterial community could be equally distributed within the two genera *Citrobacter* and *Serratia* sp. (28.3% each) on CIN. *Morganella* (n = 23, 19.2%) and *Providencia* sp. (n = 21, 17.5%) were frequently reported as well. On PCA at the 5th day, members of the genera *Pseudomonas* (n = 25, 20.3%), *Serratia* (n = 24, 20%), as well as *Proteus*, *Citrobacter*, *Morganella*, *Hafnia* and *Vagococcus* sp. were frequently identified.

Regardless of the enrichment medium, all cultures isolated from CIN were Gram-negative and, species belonging to the genera *Ewingella, Kluyvera*, and *Providencia* sp. were only recovered from this medium. On the other hand, *Acinetobacter*, *Buttiauxella*, *Empedobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Lelliotta*, *Pantoea* and *Vagococcus* sp. were only recovered from PCA plates.

Concerning *Yersinia* species, *Y. enterocolitica* and *Y. intermedia* were both retrieved from minced pork meat samples (Table 3b). In detail, in one of the samples, at the 2nd day of incubation after enrichment in PSB, two isolates from CIN were identified as *Y. enterocolitica,* and one from PCA as *Y. intermedia*. In the other sample, at the 1st day of incubation after enrichment in PSB, one colony from PCA could be ascribed to *Y. enterocolitica*, and one from CIN to *Y. intermedia*.

4. Discussion

The present work focused on the limits and weakness of conventional methods currently proposed for *Yersinia* sp. detection in pork products. SYBR Green PCR was used to detect *Yersinia* in several meat samples experimentally inoculated with pathogenic *Y. enterocolitica* biotype 4/O:3 and non-pathogenic strain 1A/O:5. Differences between the behaviour of both biotypes were observed; in fact, only the non-pathogenic *Y. enterocolitica* 1A was always detected. Moreover, the detection of pathogenic *Y. enterocolitica* 4/O:3 in samples contaminated with LC ($10–10^2$ CFU/g) levels proved to be always unsuccessful. Such outcomes are of particular concern, because in food samples, the pathogen usually occurs at low concentrations (Petsios et al., 2016). Likely, the hurdle associated to the *Y. enterocolitica* 4/O:3 isolation could originate from the small number of pathogenic cells in the sample, and the competitive large number of microorganisms of the "background flora" grown in the selective liquid medium (PSB) (Fredriksson-ahomaa and Korkeala, 2003). On the other hand, growth of biotype 1A does not seem to be affected. Therefore since *Y. enterocolitica* 4/O:3 is a weaker competitor, the other bacteria occurring in the enrichment medium may overgrow, thus prejudicing its detection (Bari et al., 2011). Furthermore, according to the present results, the temperature of incubation (10 or 25 °C) strongly affect the *Y. enterocolitica* optimal day of detection. When the incubation temperature is fixed at 25 °C, which is actually the one indicated by the ISO methods (ISO: 10273:2017), the target gene could be amplified only during the first three days of incubation, and only if the contamination level was at least of $10^2–10^3$ CFU/g (MC). This is consistent with the results of Van Damme et al. (2013). According to these authors, a two-days incubation allowed the best detection of the pathogen by culture depended method, if compared to a five-days long incubation. On the contrary, at 10 °C, and at the same inoculum size ($10^2–10^3$ CFU/g), *Y. enterocolitica* showed an opposite behaviour: the pathogen was detected in pork slices just by the day five of incubation. Since *Y. enterocolitica* is a psychrotrophic bacterium and can therefore grow at refrigeration temperature, it has been previously reported that enrichment incubation at 4 °C can improve the detection rate because the pathogen can outgrow the mesophilic flora occurring in the sample. According to the present results, with the incubation at 10 °C, the threshold of detection does not change, but a longer incubation time is needed. Actually, it is well known that at 4 °C, incubation time may last up to three weeks (Savin et al., 2012), and this represent a further disadvantage of this approach.

When naturally contaminated samples were analysed, the gene *ystA* used as target for the detection of *Y. enterocolitica* 4/O:3 was never amplified, whereas the gene *ystB*, used as target for the biotype 1A, was frequently detected. The biotype 1A has been considered as not

Figure 2

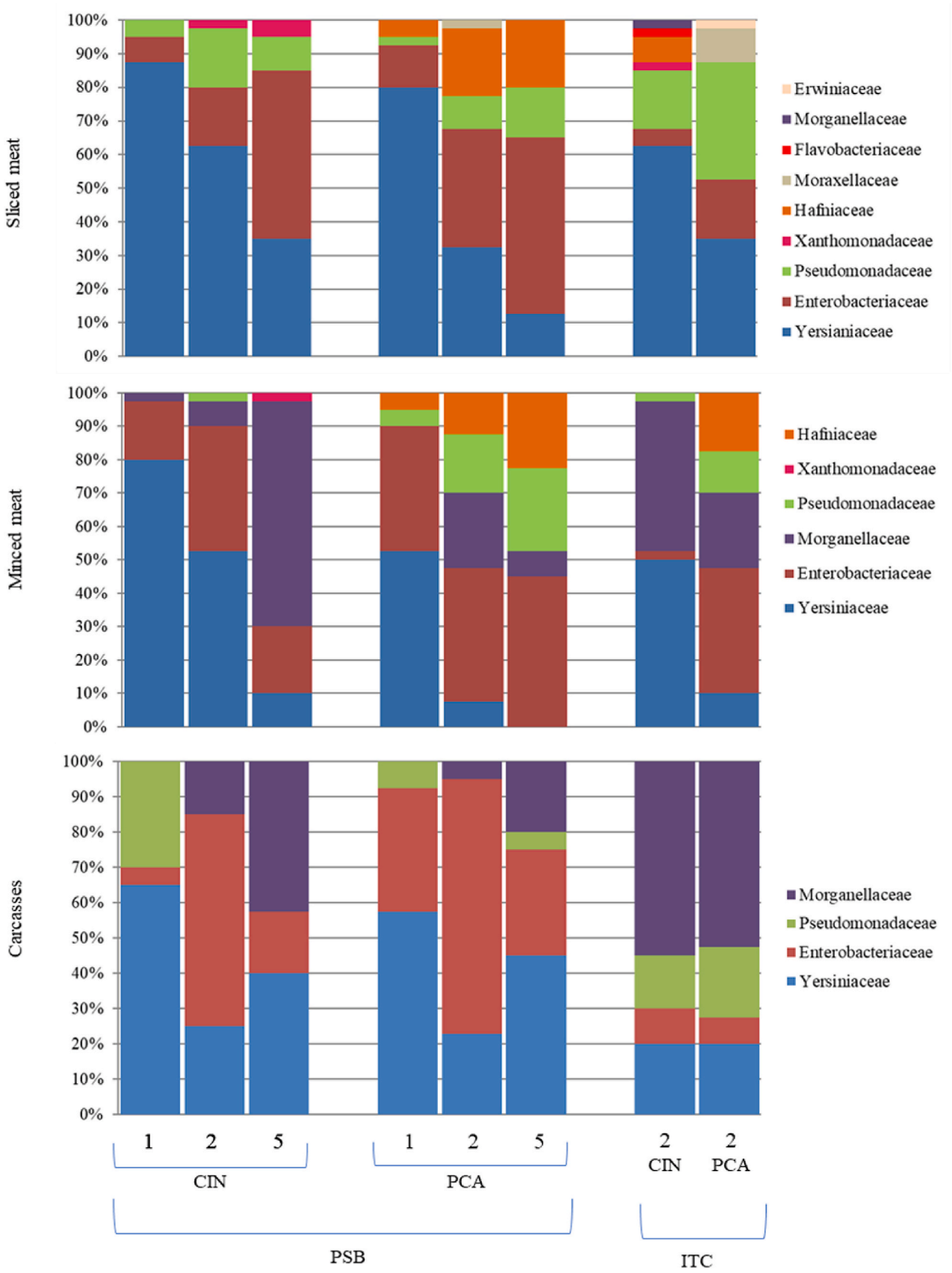

Fig. 2. Bacterial families identified on CIN and PCA by MALDI-TOF in six pork samples (2 carcasses, 2 minced meat and 2 slices) after enrichment in PSB or ITC (25 °C) for one, two and five or two days, respectively.

Table 3a
Isolates (n. 320 out of 960) identified at species level in two pork slices samples on CIN and PCA agar plates incubated at 25 °C, after enrichment in PSB and ITC for one (d-1), two (d-2) and five (d-5) days and for two (d-2) days, respectively.

		PSB						ITC	
		CIN			PCA			CIN	PCA
Genus	Species	d-1	d-2	d-5	d-1	d-2	d-5	d-2	d-2
Acinetobacter	*Guillouiae*					1			
Buttiauxella	*Agrestis*					1			1
	ferragutiae					1			
	Izardii					1			
Citrobacter	*Braakii*		2	1					
	Freundii		4	19			6	2	3
	Gillenii				3	7	1		
Empedobacter	*Brevis*						1		
Ewingella	*americana*							1	
Hafnia	*Alvei*				2	8	3		4
Klebsiella	*Oxytoca*						1		
Lelliottia	*Amnigena*					2			
Morganella	*Morganii*						1		
Pantoea	*agglomerans*								1
Pseudomonas	*azotoformans*						1		
	fluorescens		2						
	Fragi				1	2	4		
	Fulva								
	fuscovaginae					2	1		10
	Graminis						1		4
	Libanensis		1						
	rhodesiae		2	1			2		
	Synxantha	2	2	3			3		
	Taetrolens						1		
Rahnella	*Aquatilis*	3	1		2	1		1	2
Raoultella	*ornithinolytica*								1
Serratia	*Fonticola*		2			1			
	Grimesii	1	1	1					
	liquefaciens	31	21	12	32	7	6	36	14
	proteamaculans	3	1	1		5			
Stenotrophomonas	*Maltophilia*		1	2			1		
Vagococcus	*fluvialis*					1	7		

pathogenic, though in the latest years has been associated to yersiniosis cases in humans (Strydom et al., 2019). Results of the present study fully agree with previously published researches (Lucero-Estrada et al., 2020; Sirghani et al., 2018): biotype 1A is the most frequently biotype isolated and/or detected. Excluding methodological bias to be related to SYBR Green PCR, the better isolation/detection of biotype 1A, may not be necessary due to its prevalence in the sample, but rather to a greater tolerance at conditions prevailing in the enrichment broth.

To characterize the microflora occurring on CIN agar, as well as on non-selective medium PCA agar, colonies randomly picked were identified by means of MALDI TOF MS. As expected, total bacterial counts were slightly higher on PCA, but no significant difference ($p > 0.05$) could be noticed between the two media (Fig. 1). Even if bacterial loads stayed almost constant from the 2nd up to the 5th day of incubation on both CIN and PCA, the bacterial speciography noticeably changed during this time frame. In detail, Yersiniaceae was the dominant family retrieved on both CIN and PCA after enrichment in PSB at the 1st day of incubation, but as early as the 2nd day, the percentage of its isolation considerably decreased, independently by the meat sample (Fig. 2). The results of this second set of trials further confirm the poor competitiveness of members of the Yersinaceae family. Moreover, the length of the incubation appears to be a crucial aspect: the best *Yersinia* genus recovery may be obtained with an enrichment never longer than 48 h, afterward the accompanying bacterial flora in PSB broth overgrowth, thus jeopardizing the pathogen's detection. Likely, the usually adopted enrichment media, despite the presence of sorbitol and bile salts in PSB, and irgasan, ticarcillin and potassium chlorate in ITC, are not sufficiently selective, leading to growth of bacteria other than *Yersinia*, occurring in the specimen. If compared to PCA, the biodiversity of the microbial community reflects the higher selectivity of the CIN, due to the presence of cefsulodin, irgasan, and novobiosin, regardless the broth (PSB or ITC) used for enrichment (Fig. 2). However, a large number of non-*Yersinia* genera grew on CIN agar; above all several cultures belonging to *Serratia* and *Citrobacter* sp. were isolated on this medium. As matter of fact, *Serratia*, *Citrobacter, Morganella,* and *Providencia* sp. may produce colonies quite similar to *Y. enterocolitica* (Petsios et al., 2016). The species *Y. enterocolitica* and *Y. intermedia* were isolated from minced meat samples but only during the first two days of incubation in PSB. The isolation of *Y. intermedia* should not be underestimated as it is categorized as *Y. enterocolitica*-like species, and has been associated to humans and animals diseases (Imori et al., 2017).

The "accompanying flora" growing in the enrichment media used for the search of *Y. enterocolitica* was here characterized for the first time. According to data, the microbial population occurring in the enrichment media is largely affected by time and temperature of incubation, playing a crucial role in the successful detection of the pathogen. Based on the results, further inhibiting agents or selective components able to promote the recovery of *Yersinia* species, are still needed. Moreover, since the recovery of different biotypes within the genus *Yersinia* may largely vary, further experiments based on the use of additional biotypes, even as mixture, as well as of different food ecosystems, could improve the potential of Real-Time PCR as supplementary tool for the detection of this pathogen.

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Declaration of competing interest

The authors have no conflict of interest to declare.

Table 3b
Isolates (n. 320 out of 960) identified at species level in two pork minced meat samples on CIN and PCA agar plates incubated at 25 °C, after enrichment in PSB and ITC for one (d-1), two (d-2) and five (d-5) days and for two (d-2) days, respectively.

		PSB						ITC	
		CIN			PCA			CIN	PCA
Genus	Species	d-1	d-2	d-5	d-1	d-2	d-5	d-2	d-2
Buttiauxella	*gaviniae*				1				
Citrobacter	*braakii*		1						
	freundii	6	14	8	2	1	3	1	
	gillenii				1				
Hafnia	*alvei*				2	5	9		7
Klebsiella	*oxytoca*				1		1		
Kluyvera	*intermedia*	1							
Morganella	*morganii*		3	10		9	3	18	9
Proteus	*hauseri*				8	14	8		8
	vulgaris					1	2		7
Providencia	*heimbachae*	1		6					
	rettgeri			11					
	chlororaphis					3			
	cichorii					1	3		
	fluorescens								
	fragi			1		2	1		
	fuscovaginae						5		1
	kilonensis					1			4
	libanensis	1							
	lundensis			1			1		
	putida							1	
Rahnella	*aquatilis*			1					
Raoultella	*ornithinolytica*			1					
Serratia	*grimesii*							1	
	liquefaciens	5	18		4	1		7	
	plymuthica	3							
	Proteamaculans	23	1	4	16	1		12	4
Stenotrophomonas	*maltophilia*			1					
Vagococcus	*fluvialis*						4		
Yersinia	*enterocolitica*		2		1				
	intermedia					1			

Table 3c
Isolates (n. 320 out of 960) identified at species level on two pork carcasses on CIN and PCA agar plates incubated at 25 °C, after enrichment in PSB and ITC for one (d-1), two (d-2) and five (d-5) days and for two (d-2) days, respectively.

		PSB						ITC	
		CIN			PCA			CIN	PCA
Genus	Species	d-1	d-2	d-5	d-1	d-2	d-5	d-2	d-2
Buttiauxella	*Agrestis*				1				
Citrobacter	*Braakii*		4					1	1
	Freundii	1	25	6	2	1	3	3	2
Enterobacter	*Asburiae*				4				
	Cloacae				1				
	Kobei				2				
Escherichia	*Coli*				1	1	2		
Ewingella	*Americana*		3						
Klebsiella	*Oxytoca*				1		3		
Lelliottia	*Amnigena*				1				
Morganella	*Morganii*			13			8	22	21
Proteus	*Hauseri*					30	4		
	Rettgeri			4					
Pseudomonas	*Agarici*								1
	brassicacearum							1	
	Brenneri								1
	Fluorescens	3							
	Fragi						1		
	Fulva	3						1	1
	fuscovaginae				1		1		
	pseudoalcaligenes								1
	putida	6			1			4	4
	taetrolens				1				
Rahnella	*aquatilis*	1	3	1					
Raoultella	*ornithinolytica*				1				
Serratia	*grimesii*	1					1		
	liquefaciens	25	5	16	22	8	17	8	8
	proteamaculans				1				

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