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# Improved detection of *Escherichia coli* and coliform bacteria by multiplex PCR

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## Abstract

**Background:** The presence of coliform bacteria is routinely assessed to establish the microbiological safety of water supplies and raw or processed foods. Coliforms are a group of lactose-fermenting *Enterobacteriaceae*, which most likely acquired the *lacZ* gene by horizontal transfer and therefore constitute a polyphyletic group. Among this group of bacteria is *Escherichia coli*, the pathogen that is most frequently associated with foodborne disease outbreaks and is often identified by  $\beta$ -glucuronidase enzymatic activity or by the redundant detection of *uidA* by PCR. Because a significant fraction of essential *E. coli* genes are preserved throughout the bacterial kingdom, alternative oligonucleotide primers for specific *E. coli* detection are not easily identified.

**Results:** In this manuscript, two strategies were used to design oligonucleotide primers with differing levels of specificity for the simultaneous detection of total coliforms and *E. coli* by multiplex PCR. A consensus sequence of *lacZ* and the orphan gene *yaiO* were chosen as targets for amplification, yielding 234 bp and 115 bp PCR products, respectively.

**Conclusions:** The assay designed in this work demonstrated superior detection ability when tested with lab collection and dairy isolated lactose-fermenting strains. While *lacZ* amplicons were found in a wide range of coliforms, *yaiO* amplification was highly specific for *E. coli*. Additionally, *yaiO* detection is non-redundant with enzymatic methods.

**Keywords:** Multiplex PCR, Coliform detection, *Escherichia coli* identification

## Background

Public health protection requires the prompt evaluation of microorganisms in drinking water and in raw and processed foods to prevent outbreaks of microbial contamination. A broad variety of waterborne and foodborne pathogens are present at extremely low concentrations and are thus challenging to detect. Whereas coliforms [1], particularly *Escherichia coli* [2, 3], rarely cause sickness, these bacteria are abundant in human and warm-blooded animal feces and are thus regularly used as microbial indicators of the co-contaminating presence of enteropathogenic bacteria in water and foods supplies [4].

The coliform group was vaguely defined from its inception [5], primarily by the ability of bacteria to ferment lactose, and lacks taxonomical value. Coliform bacteria

are distributed among diverse genera, and different authors use different inclusion criteria [6, 7]. Nevertheless, coliforms are generally described as gram-negative, rod-shaped *Enterobacteriaceae* that ferment lactose producing acid and gas. Possession of the gene *lacZ*, which codes for the  $\beta$ -galactosidase, is the most prominent feature of the coliforms, whereas  $\beta$ -D-glucuronidase, encoded by the gene *uidA*, is routinely used to specifically identify *E. coli*. Unsurprisingly, because *uidA* and *lacZ* are paralogs [8], wild type  $\beta$ -D-glucuronidase also has a weak  $\beta$ -galactosidase activity, which is increased in some mutant alleles [9, 10]. Although lactose fermentation can be used to distinguish *Shigella* spp. from *E. coli*, several *Shigella* strains are able to ferment lactose after cultivation. This is explained by the presence of *lacZ* in specific *Shigella* genomes but the absence of LacY permease activity in culture [11].

Historically, the definition of coliforms has been primarily based on the techniques used for their detection. Traditional detection methods rely upon culturing the samples on selective media and specific incubation

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conditions [7, 12]. This approach allows cell enumeration but it is cumbersome, time consuming and fails to score viable but nonculturable (VBNC) bacterial cells. In *Enterobacteriaceae* gas formation from lactose is dependent on formic hydrogenlyase and easily inhibited [13]; thus, detection of coliforms by gas production lacks significance. This method also lacks specificity, as *Aeromonas* spp. can also ferment lactose [14]. Furthermore, both  $\beta$ -galactosidase and  $\beta$ -D-glucuronidase are inducible enzymes and their activity is affected by incubation temperature and the growth medium [1, 15, 16]. Consequently, both false-positive and false-negative bacteria interfere with evaluation. Several current commercial tests involve specific enzymatic assays that utilize chromogenic or fluorogenic substrates for the improved detection of coliforms [7, 17]. These methods are simple and rapid but their specificity is compromised [18, 19]. Although primarily limited to *E. coli* [20, 21],  $\beta$ -D-glucuronidase activity is found in other bacteria such as Flavobacteria, and it is frequent in *Yersinia*, *Salmonella*, and *Shigella* [3, 22]. Conversely, a high proportion of  $\beta$ -D-glucuronidase-negative *E. coli* strains has been reported [23, 24].

Exponential amplification of specific DNA sequences by PCR greatly increases the probability of detecting low concentrations of target organisms and reduces the time required to obtain results. While PCR cannot distinguish live from dead cells, it permits the detection of nonculturable cells. Moreover, PCR may yield positive results with strains that, despite bearing a gene sequence, do not express the corresponding enzyme and thus lack the corresponding enzymatic activity [24]. Multiplex PCR (mPCR) allows for the simultaneous evaluation of several strains and the detection of internal controls. Two sets of primers pairs, designed by Bej *et al.* [25, 26] for the amplification of *lacZ* and *uidA* genes, have been used to test for total coliforms and *E. coli* in clinical isolates [27] and in the upper gut contents of Lindow Man [28]. An mPCR assay was developed to target *uidA* gene for the common detection of *E. coli* and *Shigella* in milk [29]. Fricker *et al.* [30] analyzed water samples and found that only 70 % of the 324 coliform strains were correctly

identified by these *lacZ* primers, whereas five non-*E. coli* coliforms were identified by *uidA* primers. These results indicate that developing alternative primer sets might be required for improved detection.

In this paper, we report a new strategy for differential bacterial identification by multiplex PCR. We wanted to combine, in a single assay, wide-range and high-specificity detection of both total coliforms and *E. coli*, respectively. To overcome the variability of *lacZ* sequences, primer sets were targeted to the consensus sequence of an alignment. An orphan gene, *yaiO*, was selected as the target for the specific identification of *E. coli*. The resulting amplicons, both *in silico* and *in vitro*, indicate that these probes are more efficient than those previously described.

## Results

### *yaiO* represents an alternative to *uidA* for *E. coli* identification *in silico*

The gene *yaiO* [EcoGene:EG13297], selected as an alternative to *uid* amplicons, belongs to the *E. coli* orphan ORFs [31]. Nonetheless, *yaiO* transcribes in both the exponential and stationary growth phases [32], encodes a protein originally postulated by a bioinformatic prediction [33] and was later found to be expressed and localized in the outer membrane of *E. coli* [34]. These results indicate that *yaiO* corresponds to a bona fide gene and hence it might constitute an optimal target for specific *E. coli* identification by PCR detection. With this goal in mind, the oligonucleotide primer pair *yaiOF* and *yaiOR* was design to produce a 115 bp amplicon (Table 1).

The Primer-BLAST tool allows to check the specificity of pre-existing primers by combining local and global alignment algorithms [35]. Therefore, we used it to compare the *in silico* PCR amplification of the *uidA* primers designed by Bej *et al.* [26] (Table 1) with the *yaiO* primer set using *Enterobacteria* as the target genomes. The resulting hits were grouped by species or genus (Table 2). Unsurprisingly, because the *Shigella* and *E. coli* lineages are very closely related [11, 36], *Shigella* spp. hits were obtained for both primer sets. However, the

**Table 1** Oligonucleotide primers used for multiplex PCR amplification

Primer set	Source	Sequence	Product size (bp)
lacZB	Bej <i>et al.</i> , 1990	F: 5' ATGAAAGCTGGCTACAGGAAGGCC 3'	876
		R: 5' CACCATGCCGTGGGTTTCAATATT 3'	
lacZ3	This work	F: 5' TTGAAAATGGTCTGCTGCTG 3'	234
		R: 5' TATTGGCTTCATCCACCACA 3'	
uidA	Bej <i>et al.</i> , 1991	F: 5' TGGTAATTACCGACGAAAACGGC 3'	162
		R: 5' ACGCGTGGTTACAGTCTTGCG 3'	
yaiO	This work	F: 5' TGATTTCCGTGCGTCTGAATG 3'	115
		R: 5' ATGCTGCCGTAGCGTGTTC 3'	

**Table 2** Comparison of expected *uidA* and *yaiO* PCR products by Primer-BLAST analysis

Organism	Total number of hits		<i>yaiO</i> vs. <i>uidA</i>		
	<i>yaiO</i> +	<i>uidA</i> +	<i>yaiO</i> + <i>uidA</i> -	<i>yaiO</i> + <i>uidA</i> +	<i>yaiO</i> - <i>uidA</i> +
<i>Escherichia coli</i>	83	91	31	52	39
<i>Escherichia</i> sp.	1	2	1		2
<i>Citrobacter rodentium</i>		1			1
<i>Shigella boydii</i>	1	2		1	1
<i>Shigella flexneri</i>		6			6
<i>Shigella sonnei</i>	2	2		2	
	87	104			

The number of potential target sequences yielding positive *in silico* PCR amplification (N = 136) with *yaiO* or *uidA* primers (Table 1) are grouped by species or genus. The genomes that produce single and double amplicons are compared on the right

specificity of the *yaiO* amplification was higher 95 % of the positive hits (83 of 87) for *yaiO* primers corresponded to *E. coli*, whereas only 87.5 % of the hits (91 of 104) represented *E. coli* when the query primers were *uidA*. Ten non-*E. coli* strains rendered as hits with *uidA* primers alone, one with *yaiO*, and three were detected by both primer sets. These results suggest that although both sets are suitable for *E. coli* detection, *yaiO* might represent indeed a superior target.

#### *lacZ* alignment and primer design

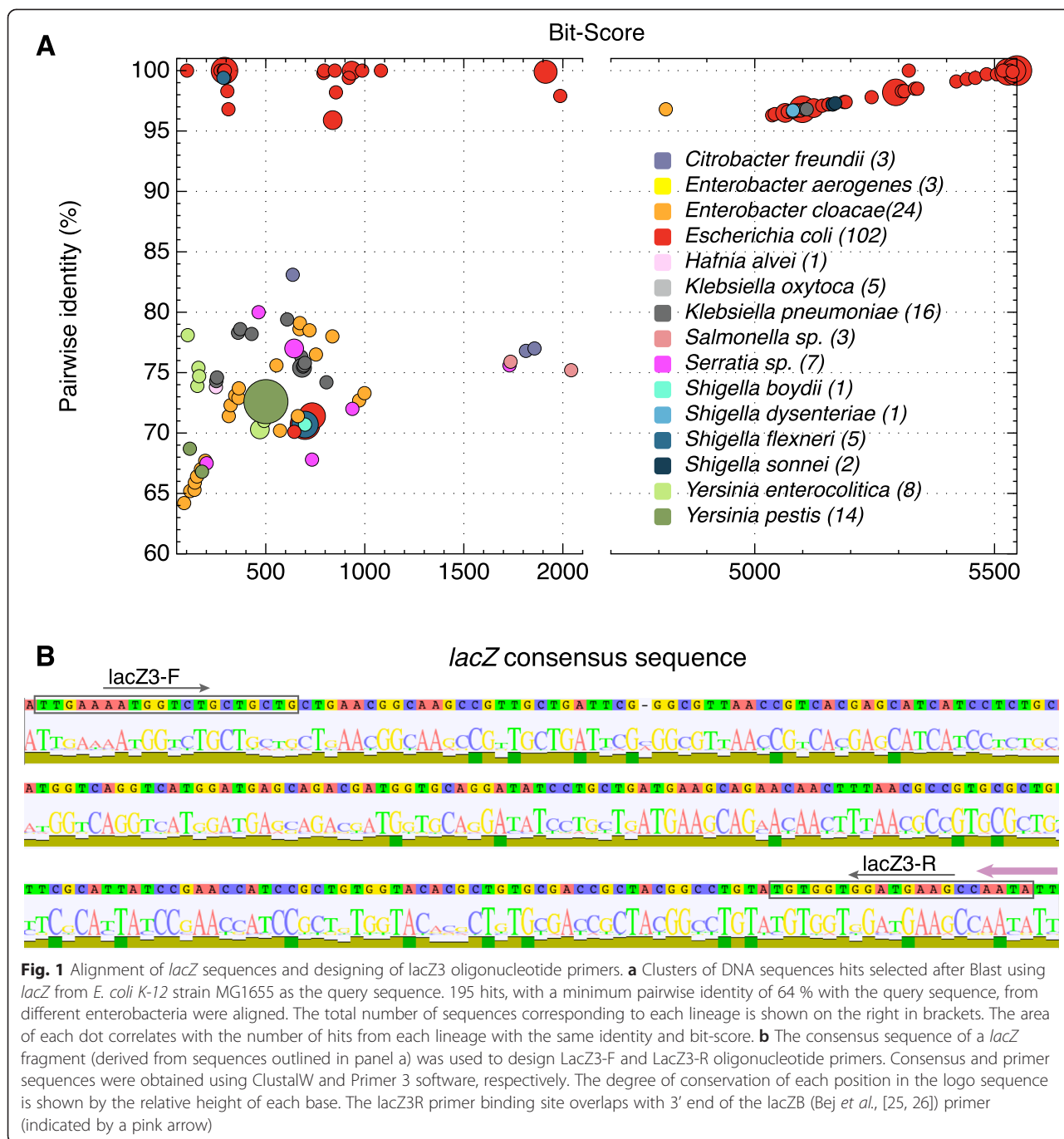
By performing a comparative analysis of an assortment of *lacZ* sequences and developing new PCR primers, it may be possible to improve the accuracy of coliform detection. The DNA sequence of the *lacZ* gene from the *E. coli* strain MG1655 [EcoGene:EG10527] was used to perform a BLASTn search. From the resulting hits, a total of 195 sequences (with a minimum identity of 64 %) were selected, and a *lacZ* consensus sequence was obtained running ClustalW [37] software. Considering the ambiguous definition of the coliform group, we did not restrict the sequences to historical coliforms but incorporated sequences from other enterobacteria (Fig. 1a). The statistical significance of the alignments was evaluated by comparing the pairwise identity (%) and the bit-scores of the selected BLAST hits, revealing three clusters of sequences. Although most *E. coli* sequences showed high identity (above 95 %), some possessed high bit-scores (above 5000), showing a linear correlation between identity and bit-score (Fig. 1a, top right), whereas others depict lower values (below 2000). These groups correspond to “full” and “incomplete”  $\beta$ -galactosidase genes, respectively. Strikingly, the bulk of the sequences from other lineages forms a third group with lower identity (below 85 %) and bit-score values. Although the lack of lactose fermentation is commonly used to distinguish *Shigellae* from *E. coli* [11, 38], some *Shigella* strains harbor the gene *lacZ* (see Background). In the analysis, BLAST hits were obtained for several *Shigella* lineages, but the hits found for *Shigella dysenteriae* and *Shigella sonnei* coincide with

the gene *ebgA*, which is paralogous with *lacZ*. These results indicate that designing PCR primers by using a *lacZ* consensus sequence as a target might widen the spectrum of coliform detection. Because similar sized amplicons are expected to work better on multiplex PCR [39], we designed the primer set to produce an amplicon that was similar in size to but distinguishable from the *yaiO* amplicon (Table 1). Therefore, *lacZ3* oligonucleotide primers (Table 1) were designed to amplify a highly conserved zone of *lacZ*, resulting in a 234 bp PCR product (Fig. 1b).

#### Specificity of *E. coli* and coliform detection using *yaiO*, *uidA* and *lacZ* primer sets for multiplex PCR

The performance of two pairs of oligonucleotide primers, named here *lacZB-uidA*, developed by Bej *et al.* [25, 26] for coliform detection by multiplex PCR, was compared with the newly designed *lacZ3-yaiO* primers (Table 3). To this end, *in vitro* multiplex PCR amplification and *in silico* PCR simulation were conducted (Materials & Methods). *In silico* evaluation is utilized to elucidate the source of false positive and false negative results obtained with *in vitro* experiments [40]. PCR reactions were carried out with culture collection and dairy isolated bacterial strains (Table 3) that had undergone total DNA extraction. Optimal thermocycling conditions with *lacZ3-yaiO* primers were determined by varying both the annealing temperature and extension time until best results were obtained (materials & methods). Amplified DNA was evaluated for the expected products using agarose electrophoresis (Fig. 2a) and compared with *in silico* simulations (Fig. 2b). Simultaneous amplifications with *lacZB-uidA* were also performed following the method described by Tantawiwat *et al.* [27]. Although described as an optimized protocol, we did not note any improvements using such thermocycling setup (data not shown).

Overall, the *lacZ3* primers showed superior identification efficiency for *E. coli* and coliform bacteria. In agreement with the *in silico* results (Fig. 2), most *E. coli* samples generated the expected PCR product with any *lacZ* primer



set, although the lacZB band was very weak or repeatability was compromised (50 % or less amplification success) in several strains, whereas the lacZ3 primers robustly identified all of the *E. coli* strains evaluated. Interestingly, additional nonspecific bands were exclusively detected when amplification was performed with lacZB-uidA and the target was not *E. coli*. Furthermore, although lacZB did not produce the expected amplicon for several coliform lineages (*Citrobacter youngae*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Salmonella typhimurium*, *Shigella*

*spp.* and *Yersinia enterocolitica*), amplification with lacZ3 resulted in the expected 234 bp band with all samples except *Shigella boydii* and *Sh. flexneri*.

### Discussion

Accurate molecular detection of *E. coli* is critical for the food industry because this bacterium is considered a primary faecal indicator. However, though it is widely assayed, neither  $\beta$ -D-glucuronidase activity nor *uidA* amplification unequivocally identify *E. coli* (see Background). Therefore,



**Table 3** Bacterial strains used in this work

Organism	Source <sup>a</sup>
<i>Escherichia coli</i> K12 (MG1655)	This Lab (Molina et al. [52])
<i>Escherichia coli</i> B (Luria)	CECT4201
<i>Escherichia coli</i> B/r	CECT105
<i>Escherichia coli</i> C (Sinsheimer)	CECT4622
<i>Escherichia coli</i> O157:H7	CECT4782
<i>Escherichia coli</i> O157:H7	CECT5947
<i>Escherichia coli</i> W (Waskman)	CECT99
<i>Escherichia coli</i> W (Stoke)	CECT727
<i>Escherichia coli</i> RT1	Torta del Casar cheese
<i>Escherichia coli</i> RT381	Torta del Casar cheese
<i>Escherichia coli</i> RT396	Torta del Casar cheese
<i>Escherichia coli</i> RT472	Torta del Casar cheese
<i>Citrobacter freundii</i>	CECT7464
<i>Citrobacter youngae</i>	CECT5335
<i>Enterobacter aerogenes</i>	CECT684
<i>Enterobacter cloacae</i> (RT102)	Ibores cheese
<i>Enterobacter intermedium</i> (RT38)	Ibores cheese
<i>Hafnia alvei</i>	CECT 158
<i>Klebsiella oxytoca</i> (RT30)	Ibores cheese
<i>Klebsiella pneumoniae</i> ssp. <i>pneumoniae</i>	CECT143
<i>Salmonella typhimurium</i>	CECT722
<i>Serratia marcescens</i> ssp. <i>marcescens</i>	CECT846
<i>Shigella boydii</i>	CECT583
<i>Shigella flexneri</i> 2a	CECT585
<i>Shigella flexneri</i> 2b	CECT4804
<i>Shigella sonnei</i>	CECT4887
<i>Yersinia enterocolitica</i> ssp. <i>enterocolitica</i>	CECT4315

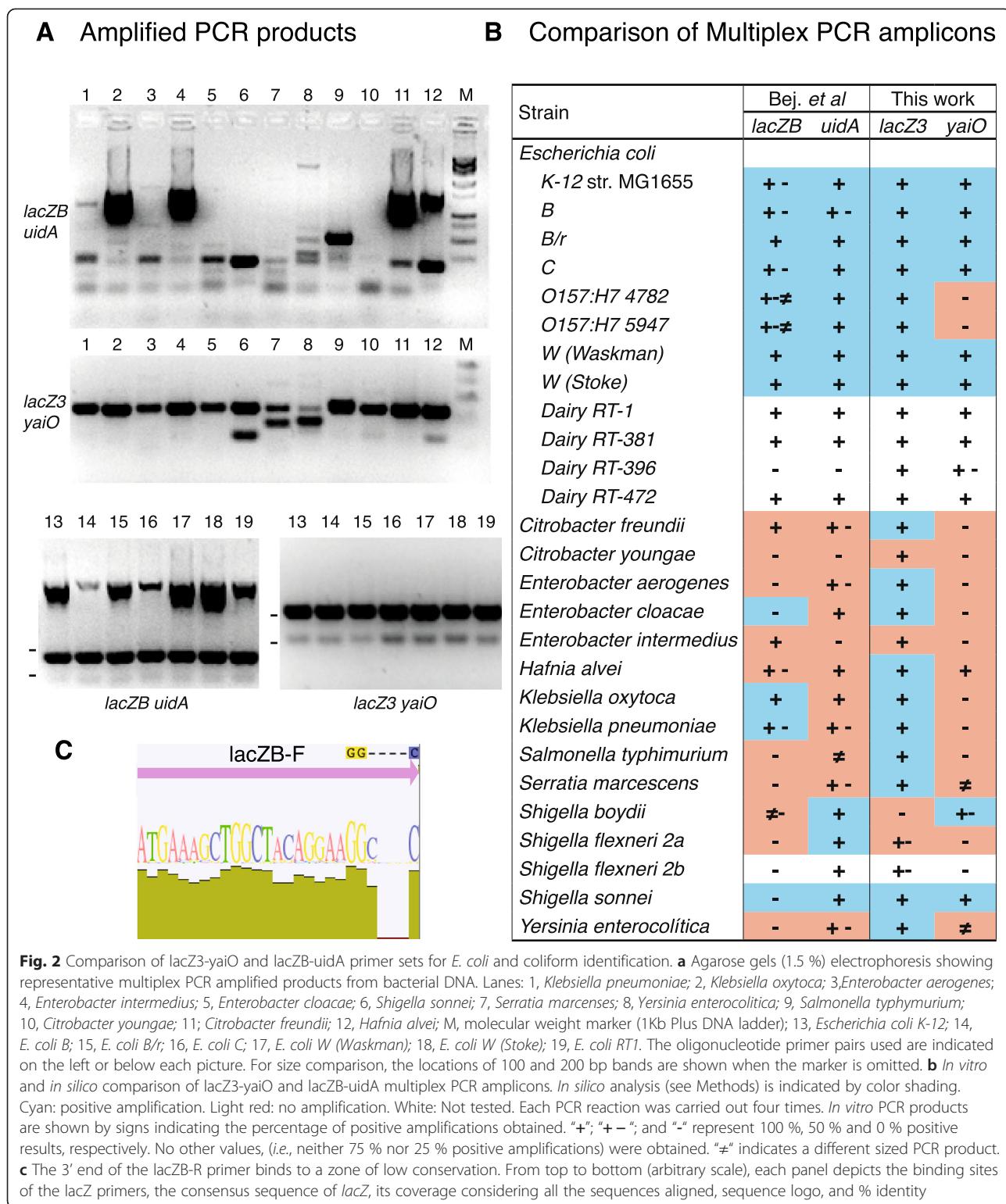
<sup>a</sup>CECT = Colección Española de Cultivos Tipo, Burjasot, Valencia, Spain  
Torta del Casar is made from raw milk of sheep from the Merino and Entrefino breeds  
Ibores cheese is made from whole, raw milk from goats of the Serrana, Verata and Retinta breeds and their crossbreeds

we wanted to design a PCR primer set non-redundant with the target of the enzymatic tests, i.e., an alternative to *uid* amplicons. To this end, we chose the gene *yaiO*, which exhibits no significant similarity to any other real or hypothetical gene [31]. Although orphan genes show a narrow distribution among *E. coli* genomes, with most of them being found in only a single genome [41], *yaiO* shows a wide within-species distribution. Essential orphan genes would be ideal targets for the highly specific identification of all *E. coli* serotypes, but comparative genomics has revealed a clear conservation tendency of essential *E. coli* genes throughout the bacterial kingdom [31]. Consequently, neither *uidA* nor *yaiO* are essential. Nonetheless, the specificity of *E. coli* identification was higher with *yaiO* primers (Fig. 2b). In agreement with our results, an evaluation of putative orthologs of *E.*

*coli* genes revealed that *yaiO* depicts a lower evolutionary retention index than does *uidA* [31], suggesting lower conservation and higher specificity of *yaiO*. Although Bej et al. [26] reported higher specificity with *uidA* primers than determined in our study, Fricker et al. [30] found false-positive *uidA* amplification using both *H. alvei* and *Serratia odorifera*. Several coliform bacteria (*Citrobacter freundii*, *Ent. aerogenes*, *Ent. cloacae*, *Klebsiella pneumoniae* and *Sh. flexneri*) produced the amplicon expected for *E. coli*, (i.e. false positives) exclusively with *uidA* primers, whereas others yielded amplicons of unexpected sizes with *uidA* (*S. typhimurium*) or *yaiO* (*Serratia marcescens* and *Yersinia pestis*). Finally, DNA from *H. alvei*, *Sh. boydii* or *Sh. sonnei* generated amplicons with both *yaiO* and *uidA* primers. Though it is considered an orphan gene, *yaiO* appears in some *Shigella* strains. However, *uidA* is present in the three *Shigella* species analyzed (Table 2 and Fig. 2b). This is not surprising because genetic variation within the four species of *Shigella* is encompassed within the range found in natural populations of *E. coli*. In fact, the *Shigella* genus has been proposed to have evolved from multiple *E. coli* strains after the divergence of the O157 and K12 lineages [42]. Our results support that the *Shigellae* should more aptly be classified as pathogenic sublineages of *E. coli* that acquired virulence factors by lateral gene transfer [43].

*E. coli* serogroup O157:H7 is the pathogen that is most commonly associated with foodborne disease outbreaks. However, O104:H4 is an emerging strain that was identified in the 2011 German epidemic [44] and could become more prevalent in the future. A +93 *uidA* single nucleotide polymorphism has been used to characterize [45, 46] and differentiate the O157 serogroup from O104:H4 [44]. Additionally, two frameshift mutations in the *uidA* structural gene account for the absence of glucuronidase activity in O157:H7 isolates [47]. Interestingly, these strains could also be differentiated by *yaiO* amplification. In agreement with the Primer-BLAST results and the absence of *yaiO* (data not shown), the O157:H7 strains did not render any PCR product with *yaiO* primers (Fig. 2b). Conversely, the O104:H4 strains harbor *yaiO* and gave positive amplification on the Primer-BLAST analysis with *yaiO* primers.

Detecting coliforms for food safety or epidemiological purposes requires an understanding of the manner in which genes are acquired and evolved because these processes may allow for the colonization of new niches and adaptation to their hosts and may possibly lead to speciation events. The lactose operon was likely acquired via horizontal transfer by unrelated bacterial lineages [48], thus hindering the establishment of alternative targets for coliform detection. Some lactose-negative strains such as *Sh. boydii* and *Sh. flexneri* lack *lacZ* but incorporate the



**Fig. 2** Comparison of lacZ3-yaiO and lacZB-uidA primer sets for *E. coli* and coliform identification. **a** Agarose gels (1.5 %) electrophoresis showing representative multiplex PCR amplified products from bacterial DNA. Lanes: 1, *Klebsiella pneumoniae*; 2, *Klebsiella oxytoca*; 3, *Enterobacter aerogenes*; 4, *Enterobacter intermedius*; 5, *Enterobacter cloacae*; 6, *Shigella sonnei*; 7, *Serratia marcescens*; 8, *Yersinia enterocolitica*; 9, *Salmonella typhimurium*; 10, *Citrobacter youngae*; 11, *Citrobacter freundii*; 12, *Hafnia alvei*; M, molecular weight marker (1Kb Plus DNA ladder); 13, *Escherichia coli* K-12; 14, *E. coli* B; 15, *E. coli* B/r; 16, *E. coli* C; 17, *E. coli* W (Waskman); 18, *E. coli* W (Stoke); 19, *E. coli* RT1. The oligonucleotide primer pairs used are indicated on the left or below each picture. For size comparison, the locations of 100 and 200 bp bands are shown when the marker is omitted. **b** *In vitro* and *in silico* comparison of lacZ3-yaiO and lacZB-uidA multiplex PCR amplicons. *In silico* analysis (see Methods) is indicated by color shading. Cyan: positive amplification. Light red: no amplification. White: Not tested. Each PCR reaction was carried out four times. *In vitro* PCR products are shown by signs indicating the percentage of positive amplifications obtained. “+”, “+ -”, and “-” represent 100 %, 50 % and 0 % positive results, respectively. No other values, (i.e., neither 75 % nor 25 % positive amplifications) were obtained. “≠” indicates a different sized PCR product. **c** The 3’ end of the lacZB-R primer binds to a zone of low conservation. From top to bottom (arbitrary scale), each panel depicts the binding sites of the lacZ primers, the consensus sequence of lacZ, its coverage considering all the sequences aligned, sequence logo, and % identity

paralogous gene *ebgA* [11], which explains the difference between the positive BLAST hits (Fig. 1a) and the negative or inconsistent amplification (Fig. 2b). Conversely, *Sh. sonnei*, which is considered lactose negative due to inactivation of the permease LacY [49, 50], harbors the

*lacZ* gene, as confirmed by amplification with lacZ3 (Fig. 2b). Similarly, *S. typhimurium* and *Hafnia alvei*, in which *lacZ* has become a pseudogene, were identified by lacZ3. Likewise, some lactose-positive *Salmonella* strains, despite being considered non-coliforms, have been found

[51]. All behavioral differences between the lacZ3 and lacZB primers pairs could be ascribed to the variability of the *lacZ* sequence, notably at the 3' end of the forward lacZB primer (relevant for the extension of the PCR), which binds to a site of low conservation in the consensus sequence of *lacZ* (Fig. 2c). On the contrary, the binding sites of both lacZ3 primers are highly conserved (Fig. 1b). Additionally, the homology between *lacZ* and *uidA* sequences and/or the size of the amplicons [39] might contribute to diminish the performance of lacZB-uidA.

## Conclusion

Horizontal gene transfer entails the high evolvability of bacterial genomes but hampers the specific detection of indicators such as coliforms. However, when the food industry involves complex bacterial activity, such as cheese production, the accuracy of the bacterial detection is crucial. Amplification of DNA sequences by PCR allows the detection of nonculturable or dead cells. The primers designed in this work, which target a conserved region of *lacZ* and the orphan gene *yaiO*, demonstrated superior detection ability when tested with laboratory collection and lactose-fermenting strains isolated from dairy samples.

## Methods

### Bacterial strains, growth conditions and DNA extraction

A total of 24 bacterial strains were used in this work (Table 3). Most reference strains were obtained from the CECT (Colección Española de Cultivos Tipo, Valencia, Spain). The *E. coli* K-12 strain belongs to our lab collection [52] (Molina et al., [52]). The rest of the strains were isolated from raw milk cheese corresponding to Torta del Casar and Queso Ibores Protected Designation of Origins. Samples were taken from milk, curd and cheese at different ripening times, and the isolates were identified (data not shown) using the Enteropluri-Test system (Liofilchem, Roseto degli Abruzzi, Teramo, Italy), the Biolog Microbial ID system (Biolog, Inc., Hayward, CA, USA), and one dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell protein [53]. All *E. coli* strains were grown at 37 °C in Lysogeny Broth medium medium, whereas other strains were cultured as described elsewhere (<http://www.straininfo.net> and <http://www.cect.org/bacterias.php>). Isolation of DNA from bacterial cells was performed using STE buffer (100 mM NaCl, 10 mM Tris buffer, pH 7.0, 1 mM EDTA) as described elsewhere [54], followed by ethanol precipitation. The concentration and quality of the DNA were determined by a spectrophotometer NanoDrop. 2000c (Thermo Fisher Scientific, Waltham, MA, USA). The DNA preparations were stored at -20 °C until use.

### Primer development

All of the oligonucleotides primers used in this study were synthesized by IDT (Integrated DNA Technologies, San Diego, CA, USA) and are listed in Table 1. Newly designed primer sequences were obtained using the Primer3 web interface [55, 56]. The DNA sequences of *yaiO* and *lacZ* genes from *E. coli* strain MG1655 were used to design *yaiO* primer pairs and as queries to perform a BLASTn analysis [57] respectively. Of the BLAST hits obtained, 195 sequences corresponding to enterobacteria were selected, and a *lacZ* consensus sequence was then determined using ClustalW [37]. The conserved regions of *lacZ* were used as template to design lacZ3 primers.

### In silico specificity test

The putative amplicons that could be generated by the *yaiO* and *uidA* primers were evaluated using the Primer-BLAST tool [35], restricting the target templates to *Enterobacteriaceae*. Low primer specificity stringency was set, and only targets with nine or more mismatches were ignored.

When available, full genome sequences of the bacterial strains shown in Table 3 were downloaded from the NCBI servers. Primer3 and MPrimer [58] were used to evaluate the *in silico* amplification with the oligonucleotide primers shown in Table 1.

### PCR optimization and conditions

All PCRs were adjusted to 50 µl with RNase-free water and contained 1 µl of 10 mM dNTP mix, 125 nM of each required oligonucleotide primer, 1.25 U of DNA polymerase (iTaq, Bio-Rad), 30 ng of template DNA and 1X PCR reaction buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl). To optimize the multiplex amplification, the concentration of MgCl<sub>2</sub> and the annealing and extension temperatures were varied (data not shown). The best results were achieved under the following conditions: 1.5 mM MgCl<sub>2</sub>, initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 58 °C for 30 s, primer extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. In every assay, a buffer control, to which no DNA template was added, was used as a negative control. To evaluate its reproducibility, all multiplex PCRs were performed four times, twice on an iCycler iQ system (Bio-Rad, Hercules, CA, USA) and twice with a Veriti-96 Well Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA). Additionally, amplifications with lacZB-uidA were carried out as described elsewhere [27]. Briefly, the thermocycling conditions were as follows: initial denaturation at 94 °C for 10 min, followed by 44 cycles of denaturation at 94 °C for 1 min, primer annealing at various temperatures (2 cycles at 62 °C, 2 cycles at 61 °C, 2 cycles at 60 °C, 2 cycles at 59 °C and 36 cycles at 58 °C) for 1 min, primer extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min.

### Detection of amplified DNA

Twenty  $\mu$ l of the PCR amplified product were separated by electrophoresis on 1.5 % agarose gel in 1X TAE buffer (40 mM Tris-base, 20 mM acetic acid, and 1 mM EDTA pH 8.0). The gel was stained with ethidium bromide (0.5  $\mu$ g/ml) or 1X SYBR Green (Life Technologies), analyzed using a GelDoc XR (Bio-Rad, Hercules, CA, USA) transilluminator and photographed with a digital camera using Quantity One 4.6.9. The 1Kb Plus DNA ladder (Life Technologies Co., Carlsbad, CA, USA) was used as a molecular marker to indicate the size of the amplicons.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

FM designed the experiments, performed the "in silico" analysis and drafted the manuscript. ELA performed the "in vitro" experiments. RT, IR and AG isolate the bacterial strains and helped to draft the manuscript. JER conceived the project, its coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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