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Short Communication

Assessment of a plasmid conjugation procedure to monitor horizontal transfer of an extended-spectrum β-lactamase resistance gene under food chain scenarios

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ABSTRACT

Plasmids are relevant reservoirs of antimicrobial resistance genes (ARGs) which confer adaptive advantages to their host and can be horizontally transferred. The aims of this study were to develop a conjugation procedure to monitor the horizontal transfer of a 193 kb plasmid containing the extended-spectrum β-lactamase production gene $bla_{CTX\text{-}M\text{-}14}$ between two *Escherichia coli* strains under a range of food chain-related scenarios, including temperature (20–37 °C), pH (5.0–9.0) or the presence of some biocidal agents (benzalkonium chloride, sodium hypochlorite or peracetic acid). The average conjugation rate in LB broth after 18 h at 37 °C was 2.09e-04 and similar rates were observed in a food matrix (cow's milk). The conjugation was reduced at temperatures below 37 °C, at alkaline pH (especially at pH 9.0) or in the presence of benzalkonium chloride. Peracetic acid and sodium hypochlorite slightly increased conjugation rates, which reached 5.59e-04 and 6.77e-03, respectively. The conjugation procedure described can be used to identify risk scenarios leading to an enhanced ARGs transmission via plasmid conjugation, as well as to identify novel intervention strategies impairing plasmid conjugation and tackling antimicrobial resistance.

1. Introduction

The great importance of antimicrobial resistance (AMR) for public health is widely recognized (Capita and Alonso-Calleja, 2013). Nowadays, around 700,000 deaths per year are globally associated with AMR in bacteria, and this figure is predicted to increase to up to 10 million deaths in 2050 (O'Neill, 2014). There is a growing concern over the possibility of AMR transmission via the food chain (Oniciuc et al., 2019; Verraes et al., 2013). Indeed, microorganisms occurring in food and food-related environments are important reservoirs of antimicrobial resistance genes (ARGs) and can be facilitators for their dissemination to the human population (Wang et al., 2006). In food-related settings, microbial biofilms, complex communities which colonize niches difficult to clean and disinfect, are of particular relevance as AMR reservoirs and hotspots for the interexchange of ARGs among microorganisms (Dass and Wang, 2022). Therefore, it is of utmost importance to understand the routes and mechanisms implicated in AMR dissemination from farm to fork. Keeping food safe for consumers relies to a large extent on the use of different strategies like industrial cleaning and disinfection with biocides (Buffet-Bataillon et al., 2016; Whitehead et al., 2011), alternative agents for biofilm removal (Liu et al., 2014), bacterial inactivation processing technologies (Barba et al., 2017), and preservatives with antimicrobial properties (Pisoschi et al., 2018).

Foods (Guillén et al., 2021) and food processing environments (Alvarez-Ordóñez et al., 2015) are complex environments in which microorganisms may face natural stress conditions such as adverse pH, osmolarity, oxidation, and extreme temperatures, among others. However, these harsh conditions or antimicrobial agents can exert a selective pressure favoring isolates with enhanced AMR, which harbor resistance determinants, transferred on some occasions by horizontal gene transfer (HGT) (Webber et al., 2015). Some niches along the food chain can be considered as HGT hotspots, where high microbial densities and the occurrence of stress conditions can favour the transfer of mobile genetic elements and ARGs between donor and competent recipient cells (Kelly

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et al., 2009; Rossi et al., 2014). The resultant transconjugant populations are better adapted to survive in the harsh environments of the food chain and may be equipped with a set of genes related to virulence and/or AMR which may pose emerging risks to consumers (Skippington and Ragan, 2011). Remarkably, recent findings indicate that some non-antibiotic drugs and antimicrobial agents can significantly accelerate the conjugation of plasmid-borne ARGs in bacterial communities (Wang et al., 2019). It is still controversial whether such induction of ARGs transmission occurs in food industry settings. Nevertheless, intervention strategies and agents designed to minimize plasmid conjugation hold promise to improve food safety, acting as secondary barriers to the spread of AMR in food-related settings.

The main objectives of this study were to validate a procedure to easily monitor in liquid systems the HGT of a plasmid harboring the extended spectrum β-lactamase production (ESBL) gene *bla*$_{CTX\text{-}M\text{-}14}$ in *Escherichia coli* and to use it to quantify AMR transfer rates under different scenarios of relevance for the food chain, i.e., various temperatures, pHs, presence of biocidal agents at sub-lethal concentrations, and in a food matrix. ESBL producing *Enterobacteriaceae* have been categorized as critical by the World Health Organization (World Health Organization, 2017) and the importance of ESBL producing *E. coli* is also supported by other scientific studies, which have pointed out to an increased prevalence of these AMR pheno- and geno-types both in health-care (Larramendy et al., 2020) and food-chain related settings (Bergšpica et al., 2020).

2. Materials and methods

2.1. Bacterial strains

E. coli HV40, the strain used as a donor strain, was kindly provided by the research group SAMA of Universidad de León. The strain was initially isolated from goat milk during a screening of ESBL-producing *Enterobacteriaceae* in milk and dairy products, and harbors a 193 kb conjugative plasmid which carries, among others, the *bla*$_{CTX\text{-}M\text{-}14}$ gene (Supplementary Fig. 1) (Alegría *et al.*, 2020). Four *E. coli* strains from the Spanish Type Culture Collection ("Colección Española de Cultivos Tipo", CECT) were initially considered as possible recipient strains (CECT-99; CECT-515; CECT-516; CECT-4267). Strains were stored at −20 °C in cryopreservant nutritive media (Cryoinstant, VWR International, Llinars del Vallés, Spain). Luria-Bertani (LB) (Merck Sigma-Aldrich, Darmstadt, Germany) was used as nutritive media (as broth or after supplementation with bacteriological agar) to grow the bacteria during the experiments.

2.2. Antibiotics and biocides

Cefotaxime and streptomycin (Merck Sigma-Aldrich) were used during the conjugation experiments. For antimicrobial susceptibility testing (AST), Sensititre Gram Negative GNX3F plates (Thermo Fisher Scientific, Oregon, USA) were selected to determine the minimum inhibitory concentrations (MICs) for the *E. coli* strains of different antibiotics. Three biocides were tested during plasmid conjugation: benzalkonium chloride (BAC) (Sigma Aldrich, St. Louis, USA), peracetic acid (PAA) (Sigma-Aldrich, Steinheim, Germany), and sodium hypochlorite (NaClO) (Honeywell, Seelze, Germany).

2.3. Selection of strains and antibiotics for the conjugation procedure

Bacterial MICs, defined as the lowest antibiotic concentrations (μg/mL) where no growth was appreciated after 24 h of incubation at 37 °C, were obtained for the five *E. coli* strains from Sensititre 96-well GNX3F plates. For that, briefly, for each strain, a 0.5 McFarland suspension was prepared in sterile distilled water, and 30 μL of this suspension was diluted in 11 mL of Mueller Hinton broth (Merck), which was used to fill in the Sensititre panel plates (50 μL per well). Growth was visually determined after incubation for 24 h at 37 °C.

From these preliminary trials, *E. coli* CECT-516, cefotaxime as a β-lactam antibiotic and streptomycin as a non-β-lactam antibiotic were selected to detect possible transconjugants attending to the requirements specified by Leungtongkam et al. (2018): the donor strain should have higher resistance to β-lactam antibiotics than the recipient strain, while it should be more susceptible to a non-β-lactam antibiotic than the recipient strain. Colonies able to grow in agar plates supplemented with both antibiotics (the β-lactam and the non-β-lactam antibiotic) were considered successful recipient bacteria that captured the plasmid.

Although the susceptibility of the donor strain to streptomycin was higher than that of *E. coli* CECT-516, the differences were small (2-fold), which hampered the discrimination on selective solid media of donor, recipient and transconjugant bacteria. Therefore, a recipient variant strain adapted to streptomycin was developed through successive culturing of *E. coli* CECT-516 with increasing concentrations of this antibiotic following the protocol described by Yen and Papin (2017). Briefly, a 0.5 McFarland suspension was prepared and 30 μl of it were diluted into 11 mL of fresh LB. 100 μL of this bacterial suspension were added as inoculum to each of the wells of a 96-well plate containing 100 μL of LB supplemented with 2-fold increasing concentrations of streptomycin. After 24 h of incubation at 37 °C, 40 μL from the well with the highest antibiotic concentration and visible growth were diluted in 10 mL of fresh LB, serving to inoculate a new 96-well plate. This process was repeated for 4 cycles, which allowed to increase the MIC from 32 μg/mL in *E. coli* CECT 516 to 2048 μg/mL in *E. coli* CECT 516strR.

To select the most appropriate antibiotic concentrations for the enumeration of transconjugants in the conjugation experiments, LB agar plates supplemented with different concentrations of cefotaxime and/or streptomycin were plated in triplicate with 100 μL of different serial dilutions of donor and recipient cultures incubated for 18 h at 37 °C.

2.4. Conjugation protocol

The protocol followed to obtain transconjugants is summarized in Fig. 1. Briefly, 100 μL of the stocks of both donor and recipient strains were inoculated in 10 mL of LB broth supplemented with cefotaxime (4 μg/mL) or streptomycin (32 μg/mL), respectively, in order to avoid a loss of antibiotic resistance. After 24 h incubation at 37 °C, grown cultures were inoculated in a ratio of 100 μL of inoculum/10 mL of fresh LB supplemented with the corresponding antibiotic. Once absorbance at 610 nm (spectrophotometer UV-3100PC, VWR) reached a value of 0.30 ± 0.05, 30 mL of bacterial cultures were centrifuged (Centrifuge 5804R, Eppendorf, Hamburg, Germany) for 5 min at 10,000 rpm. Afterwards, a washing step was performed twice by adding 10 mL of fresh LB. Finally, bacterial pellets were resuspended in 5 mL of LB broth, and 200 μL of donor and recipient suspensions were inoculated in the same tube (co-culture) and incubated at 37 °C for 18 h. 100 μL of decimal serial dilutions in LB of this co-culture were spread on LB agar plates supplemented with cefotaxime alone (16 μg/mL), streptomycin alone (32 μg/mL) or their combination for donor, recipient and transconjugant colonies enumeration, respectively. Independent grown cultures of donor and recipient strains served as controls. Conjugation rates were calculated as the number of transconjugants divided by the number of recipient colonies.

Confirmation that transconjugant colonies belonged to the recipient strain was performed through matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Microflex LRF, Bruker Daltonic, Germany), following the manufacturer's "Ethanol/Formic Acid Extraction Sample Preparation Procedure", while the acquisition of the gene *bla*$_{CTX\text{-}M\text{-}14}$ by recipient transconjugants was confirmed by PCR on various transconjugant colonies per conjugation experiment. An additional phenotypic confirmation was effectuated by streaking presumptive donor, recipient, and transconjugant colonies on LB agar plates supplemented with cefotaxime and streptomycin.

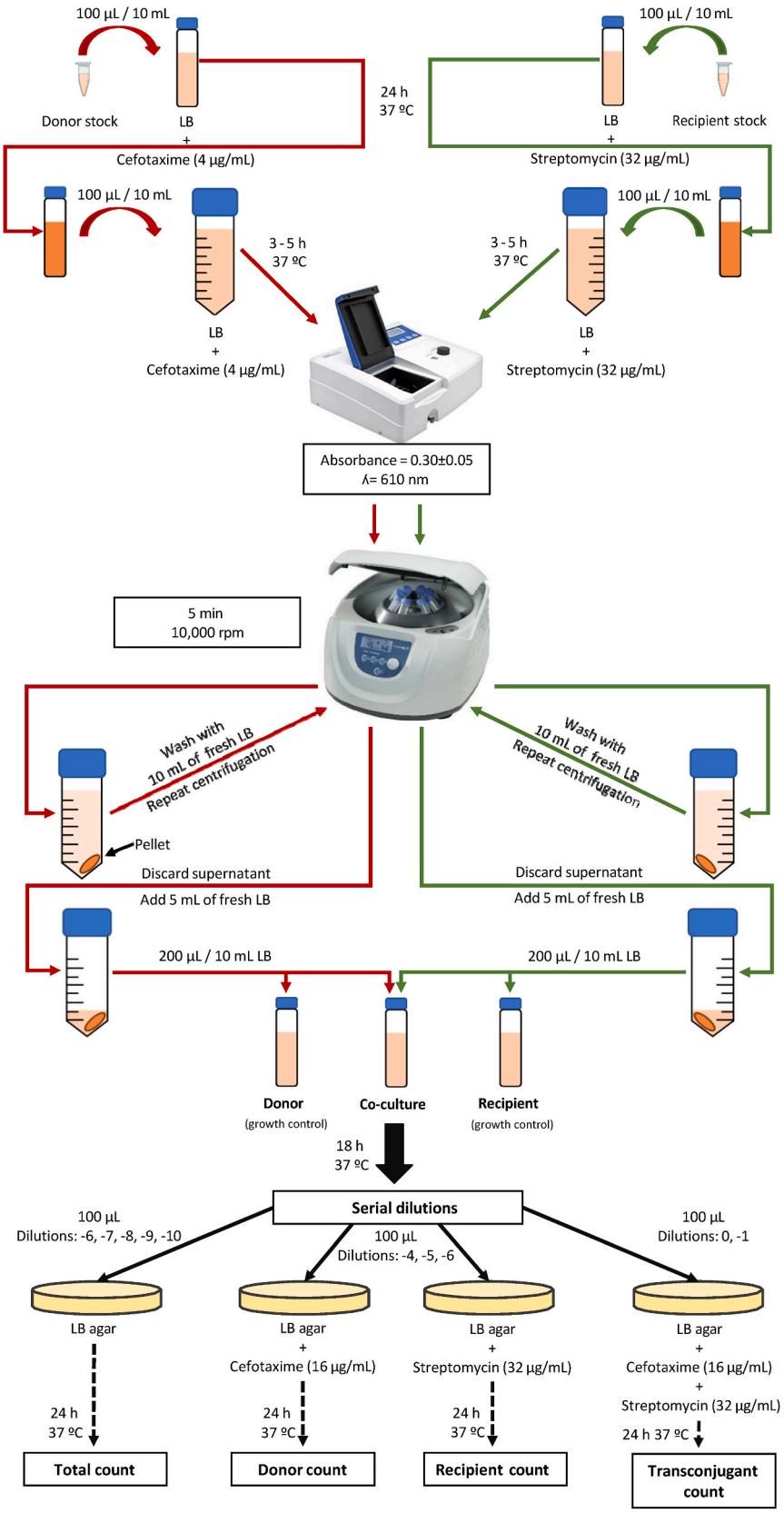

Fig. 1. Schematic representation of the conjugation protocol: Arrows show steps carried out with the donor (red arrows) and recipient (green arrows) strains, respectively. Bacteria were initially grown on 10 mL of LB broth. To obtain cultures in the exponential phase of growth, 400 µl of a grown broth culture were inoculated in tubes filled in with 40 mL of fresh LB supplemented with antibiotics, and optical density was monitored at 610 nm.

For MALDI-TOF MS, the creation of Main Spectra Profiles (MSP) was performed in a similar way to that described by Assis et al. (2017), with the minor modification that 6 fire bursts of 40 shots were applied to each well. MSP were created from 8 spectra replicates, and served to compare the transconjugants' protein profile to that of donor and recipient MSP. Molecular confirmation of $bla_{CTX\text{-}M\text{-}14}$ presence was performed by PCR following the method detailed by Pallecchi et al. (2004). Briefly, primers (Merck Sigma-Aldrich) CTX-MU1 (5′-ATGTGCAGYACCAGTAARGT) and CTX-MU2 (5′-TGGGTRAARTARGTSACCAGA) served to amplify a conserved region of bla*CTX-M* genes. An initial step of 5 min at 94 °C was followed by 35 cycles of denaturation for 45 s at 94 °C, annealing for 45 s at 56 °C, and elongation for 45 s at 72 °C, finalizing with a 10 min final extension at 72 °C. Amplification products were revealed in an agarose gel confirming the presence of $bla_{CTX\text{-}M\text{-}14}$ in donor and transconjugants colonies.

2.5. Effect of temperature, pH, presence of biocides, and food matrix on conjugation efficiency

As a proof of concept, the conjugation procedure developed was followed to monitor conjugation rates obtained under different food chain-related conditions, including: incubation temperatures of 20 °C, 30 °C and 37 °C, pH values of 5.0, 6.0, 7.0, 8.0 and 9.0, adjusted using hydrochloric acid (VWR international) or sodium hydroxide (Panreac, Barcelona, Spain) 1 N solutions, and the biocides benzalkonium chloride (BAC), peracetic acid (PAA), and sodium hypochlorite (NaClO) at sub-inhibitory concentrations. The MICs of these three biocides were determined for the donor and recipient strains using the broth micro-dilution method. The maximum non-inhibitory concentrations, which were 0.003% PAA, 8 μg/mL BAC and 2500 μg/mL NaClO, were separately added to LB broth during co-culture to evaluate their effect on plasmid conjugation.

The conjugation experiments were also replicated under control conditions in ultra-high-temperature (UHT) processed cow's milk as a food matrix.

2.6. Statistical analysis

Significant differences in conjugation rates were determined using the function *pairwise_wilcox_test* from *rstatix* R package under R version 4.0.3. Bonferroni correction for multiple comparisons was applied to *p*-values.

3. Results and discussion

3.1. Selection of recipient strain and antibiotics for the optimization of the conjugation procedure

The susceptibility to a range of antibiotics from different antimicrobial families of the donor strain, *E. coli* HV40, and the four potential recipient strains, *E. coli* CECT-99, CECT-515, CECT-516, and CECT-4267, were evaluated using a Sensititre generic panel (Table 1). *E. coli* HV40 was more resistant than the collection strains not only to all β-lactam antibiotics included in the panel but also to most of the non-β-lactams, such as ciprofloxacin, colistin, minocycline or tobramycin.

E. coli CECT-516 was selected as the recipient strain as it was the only strain showing a higher MIC (4 μg/mL) than the donor strain (2 μg/mL) to one of the tested antibiotics (the aminoglycoside gentamycin), while it maintained a lower resistance to β-lactams (e.g., the MIC of cefotaxime was 2 μg/mL and 64 μg/mL for *E. coli* CECT-516 and *E. coli* HV40, respectively). Subsequent experiments on LB agar plates confirmed that *E. coli* CECT-516 was also more resistant to other aminoglycosides, such as streptomycin, than the donor strain. In fact, the MIC of streptomycin was of 32 and 16 μg/mL for *E. coli* CECT-516 and *E. coli* HV40, respectively. Nevertheless, that small difference was not enough for a complete discrimination of the donor, recipient and transconjugant strains relying on their capability to grow in the different agar plates supplemented with antibiotics. The sequential adaptation of *E. coli* CECT-516 to streptomycin yielded a variant strain (*E. coli* CECT-516strR) with a streptomycin MIC of 2048 μg/mL that allowed to successfully differentiate between donor, recipient, and transconjugant colonies. Subsequent experiments on agar plates evidenced that a concentration of 16 μg/mL cefotaxime and 32 μg/mL streptomycin when combined together completely inhibited the growth of both strains individually inoculated, even with high microbial loads, while when used individually allowed the growth to high numbers of the donor and recipient strain, respectively.

Table 1
Minimum inhibitory concentrations (in μg/mL) of the antibiotics in the Sensititre panel GNX3F for the donor and candidate recipient *E. coli* strains.

Antibiotic	*E. coli* CECT 99	*E. coli* CECT 515	*E. coli* CECT516 (WT Recipient)	*E. coli* CECT 4267	HV40 (Donor)
Amikacin	4.00	4.00	4.00	4.00	8.00
Ampicillin/ sulbactam 2:1 ratio	4.00	4.00	4.00	4.00	16.00
Aztreonam	2.00	4.00	2.00	2.00	16.00
Cefepime	2.00	2.00	2.00	2.00	32.00
Cefotaxime	2.00	2.00	2.00	2.00	64.00
Ceftazidime	1.00	2.00	1.00	1.00	2.00
Ciprofloxacin	0.01	0.25	0.25	0.01	0.50
Colistin	0.25	0.50	0.50	0.50	2.00
Doripenem	0.50	0.50	0.50	0.50	1.00
Gentamicin	1.00	1.00	4.00	1.00	2.00
Imipenem	1.00	1.00	1.00	1.00	1.00
Levofloxacin	1.00	1.00	1.00	1.00	1.00
Meropenem	1.00	1.00	1.00	1.00	1.00
Minocycline	4.00	2.00	4.00	4.00	16.00
Piperacillin/ tazobactam constant 4	8.00	8.00	8.00	8.00	8.00
Tigecycline	0.25	0.25	1.00	0.50	1.00
Tobramycin	1.00	1.00	1.00	1.00	2.00

"WT Recipient" is the abbreviation of "Wild-type Recipient".

3.2. Influence of temperature, pH and food-matrix on the efficiency of plasmid conjugation

Control conditions for conjugation were set at pH 7, 37 °C, and absence of biocides. This control condition was always tested in parallel to the different conjugation experiments in changing environmental conditions during co-culture of donor and recipient strains in LB broth. The conjugation rate (confidence interval at 95%) under control conditions ranged between 0.64e-04 and 3.54e-04. Confirmation of transconjugants was completed by streaking various transconjugant colonies on agar plates supplemented with cefotaxime and streptomycin (Supplementary Fig. 2), as well as through MALDI-TOF spectrometry characterization and molecular confirmation of $bla_{CTX\text{-}M\text{-}14}$ by PCR (data not shown).

It has been previously reported that conjugation rates depend on many factors. Some of the biological factors include donor and recipient strains relatedness, or the type of mobile genetic element (MGE). Recipient strains already harbouring plasmids may not be competent to integrate new ones if they belong to the same plasmid incompatibility group (Thomas, 2014). Moreover, certain physichal-chemical factors like salinity, pH, shaking conditions, and type of media (agar or broth) during co-culture can also have a marked influence (Alderliesten et al., 2020). Temperature affected conjugation rates (Fig. 2A, E). Thus, transconjugants were only detected in conjugation experiments carried out at 37 °C (Fig. 2A). Similar results have been reported by Headd and Bradford (2018), who obtained very low conjugation rates for *E. coli* and the plasmid pUUH239.2 (harboring genes of resistance to β-lactams,

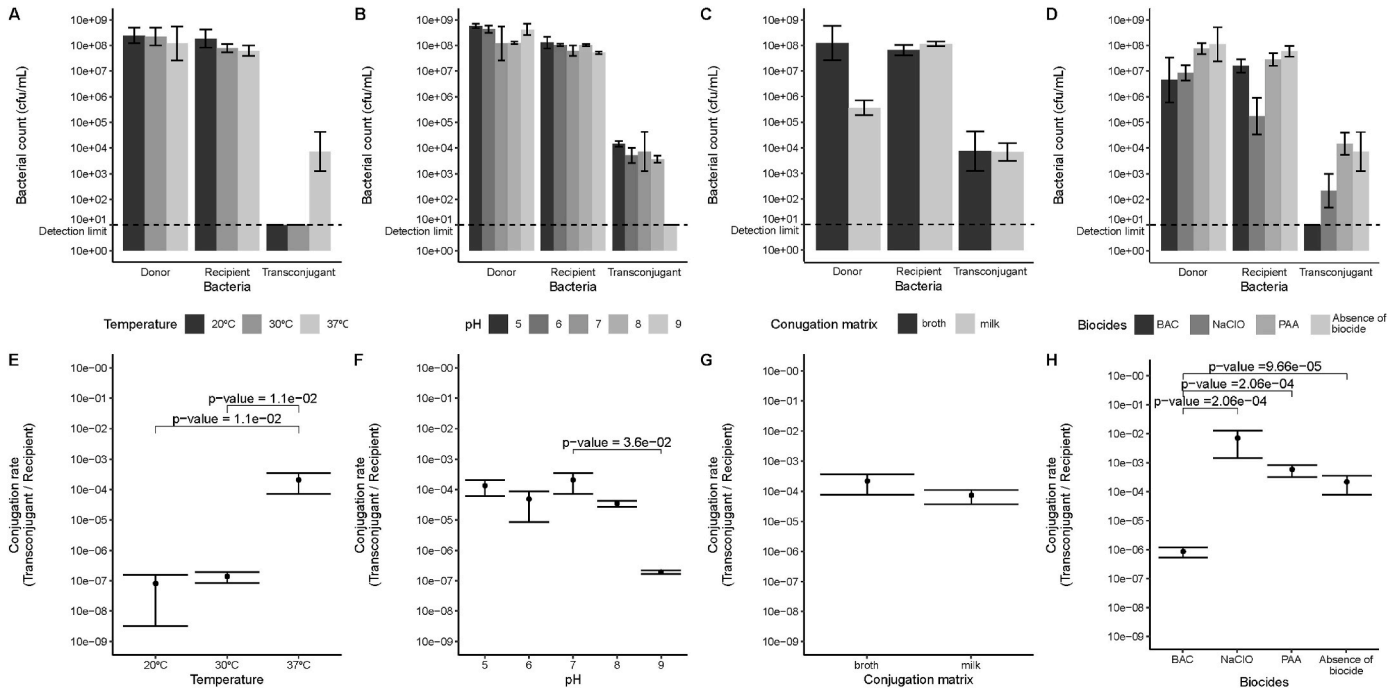

Fig. 2. Conjugation rates under different conditions: Bacterial counts (upper row) and average conjugation rates with their 95% confidence interval (bottom row) obtained when the conjugation protocol was applied under different conditions of temperature (first column), pH (second column), conjugation matrix (third column), or presence of biocides (fourth column). The dashed line represents the lower limit of detection. BAC = Benzalkonium chloride, NaClO = sodium hypochlorite, PAA = peracetic acid, cfu = colony forming units. In those cases were no transconjugants were detected, conjugation rates were estimated using the limit of enumeration of the transconjugants detection method (10 cfu/mL).

tetracycline and kanamycin) at temperatures below 37 °C, while maximum conjugation rates were reached at 37 °C. These authors concluded that conjugation occurs just in a specific window of time, when donor cells are growing and recipient cells are transitioning to a nongrowing phase. This window of time may likely take place at different incubation times depending on the growth temperature, and this could be the reason why no conjugation was observed in our study at the lowest temperatures tested. Nonetheless, these observations do not agree with results obtained by Alderliesten et al. (2020), who, in 32 studies covering 313 conjugation frequencies, showed that, in general, higher conjugation rates were reached at temperatures between 20 °C and 30 °C (1.15 times higher than those obtained at 35–37 °C). However, the use of plasmids from different incompatibility groups or bacterial species with different optimum growth temperatures makes it difficult to establish general conclusions on the effect of temperature on the HGT of MGEs.

Regarding pH, conjugation rates decreased as pH increased. Thus, the conjugation rates observed at pH 8.0 were 1.4 to 6-fold lower than those obtained in the pH range 5.0–7.0, and a more evident reduction was even seen at pH 9, where plasmid conjugation was completely inhibited (Fig. 2B, F). These results agree with those previously reported by Toomey et al. (2009), who observed that alkaline conditions (pH 8) decreased or completely inhibited horizontal transfer between *Lactococcus lactis* strains of MGEs harboring the resistance genes *tetM* and *ermB*. This reduction in the conjugation efficiency at extreme alkaline conditions could be due to a reduction in the *pilus* adhesion and stability, as reported by Klinth et al. (2012).

Conjugation rates (confidence interval at 95%) obtained in UHT milk under control conditions ranged between 3.33e-05 and 1.07e-04, not showing significant differences (*p-value* = 0.697) with those found in LB broth (Fig. 2C, G). Both milk and non-selective nutritive media like LB broth are matrixes rich in nutrients, with neutral pH, and a high water activity, and therefore suitable for the growth of bacteria. Thus, it is expected that the donor and recipient strains will have similar metabolic states for conjugation to occur in both matrixes.

3.3. Influence of type of biocide on the efficiency of plasmid conjugation

In relation to the effect of biocides, BAC was revealed as the most effective biocide to inhibit plasmid transfer. Indeed, the presence of BAC in the co-culture broth medium entirely restrained occurrence of transconjugants (Fig. 2D). On the other hand, conjugation rates were maximum in the presence of NaClO, mainly due to a ~2 log reduction in the recipient's population. In fact, they were 32-fold higher than those obtained under control conditions (Fig. 2H). Conjugation rates obtained in the presence of PAA were subtly higher than those observed under control conditions (Fig. 2H). It is generally accepted that the presence of antibiotics may induce conjugation, also of plasmids encoding ESBL production genes (Liu et al., 2019), although this effect may depend upon the antibiotic family, as it was observed for just one of seven antibiotics tested in the work of Headd and Bradford (2018). On the other hand, the effects of the presence of other non-antibiotic agents with antimicrobial activity have been less studied. However, based on previous knowledge, sub-inhibitory concentrations of biocides, like chlorhexidine, digluconate or triclosan, can increase horizontal transfer events (Curiao et al., 2016). Results obtained with the protocol developed in our study evidence that some biocides can inhibit HGT of some MGEs when used at subinhibitory concentrations, as in the case of BAC.

4. Conclusions

To conclude, we have established an operative procedure to test the impact in liquid systems (LB broth and cow's milk) of different environmental parameters on the transmission *via* plasmid conjugation of an ESBL resistance gene between *E. coli* strains and we have shown that temperatures in the range of 20–30 °C, alkaline pH values and some biocides, such as BAC, can reduce conjugation events involved in its spread, while the presence of NaClO may induce them. The results here

obtained should be taken with caution and are just relevant to the conditions being tested (liquid mating without shaking between related strains of *E. coli*), as the conjugation is affected by many factors, as evidenced in the meta-analysis undertaken by Alderliesten et al. (2020): plasmid incompatibility group, relatedness between donor and recipient strain, solid *vs* liquid mating, or agitation of the medium, among others. Nonetheless, the conjugation procedure here developed can be used in the future to identify risk scenarios leading to an enhanced ARG transmission via plasmid conjugation, or to identify agents that can impair it and are, therefore, good candidates to be incorporated into novel intervention strategies for tackling AMR.

CRediT authorship contribution statement

Adrián Alvarez-Molina: performed the lab work, analysed the results, wrote the draft manuscript. **Elena Trigal:** performed the lab work. **Miguel Prieto:** designed the experiment. **Mercedes López:** designed the experiment. **Avelino Alvarez-Ordóñez:** designed the experiment, analysed the results, wrote the draft manuscript, all authors revised and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2022.11.024.

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