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Antibiotic resistance in the viral fraction of dairy products and a nut-based milk

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ABSTRACT

Phages, the most abundant biological entities in the biosphere, can carry different bacterial genes, including those conferring antibiotic resistance. In this study, dairy products were analyzed by qPCR for the presence of phages and phage particles containing antibiotic resistance genes (ARGs). Eleven ARGs were identified in 50 samples of kefir, yogurt, milk, fresh cheese and nut-based milk (horchata), purchased from local retailers in Barcelona. Propagation experiments showed that at least some of the phages isolated from these samples infected *Escherichia coli* WG5, which was selected as the host strain because it does not contain prophages or ARGs in its genome. Electron microscopy revealed that the phage particles showed morphologies compatible with the *Myoviridae* and *Siphoviridae* families. Our results show that dairy products contain ARGs within infectious phage particles and may therefore serve as a reservoir of ARGs that can be mobilized to susceptible hosts, both in the food matrix and in the intestinal tract after ingestion.

1. Introduction

Antibiotic resistance is recognized as a worldwide threat to human health. The overuse of antibiotics in human and veterinary medicine has led to the emergence of antibiotic resistances and epidemiological studies verify that antibiotic consumption is directly connected with this emergence and the dissemination of resistances (More, 2020; Ventola, 2019).

In livestock, although their application as growth supplements has been banned in Europe since 2006 (The European Parliament and the Council of the European Union, 2003), antibiotics are still widely used for prophylaxis and/or treatment of infectious diseases (Cabello, 2006; Kemper, 2008). The continuous exposure of animals to antibiotics promotes the development of antimicrobial resistance. Under persistent selective pressure, commensal bacteria in livestock become reservoirs of antibiotic resistance genes (ARGs), which can be transferred to pathogenic bacteria. Resistant bacteria can be transmitted through the consumption of meat and other livestock products (e.g., milk and eggs) and be disseminated in the environment via animal wastes (Gomes and Henriques, 2016; Manyi-Loh et al., 2018). Additionally, antibiotic residues have been detected in animal tissues and milk (Landers et al., 2012; Sachi et al., 2019).

Agents of antimicrobial resistance, including ARGs, have been reported in different genera of lactic acid bacteria (LAB) isolated from dairy products, where they are the most abundant microorganisms (Erginkaya et al., 2018; Wang et al., 2019). Although most LAB strains are not pathogenic, the ARGs they harbor may be disseminated to other microorganisms. In addition, antibiotic resistance has been detected in viromes isolated from the air environment of dairy processing plants as well as in agricultural slurry (Colombo et al., 2018; Cook et al., 2021), which therefore represent additional reservoirs of antimicrobial resistance.

Antibiotics are naturally produced by microorganisms as a protective mechanism against competitors (Checcucci et al., 2020). While resistance to antibiotics is intrinsic to bacteria, it may also be acquired by mutation or horizontal gene transfer (Von Wintersdorff et al., 2016). The most studied mechanisms of DNA mobilization are conjugation and transformation, but transduction events or gene transfer mediated by phages have been recently revealed as another important route (Balcázar, 2020; Lekunberri et al., 2017).

Bacteriophages are the most abundant and ubiquitous biological entities in the world, outnumbering bacteria by an estimated ten-fold (Suttle, 2007). They are present in every ecosystem where bacteria exist (Pujato et al., 2019) and have a long persistence in the

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environment. Phage resilience facilitates the diffusion of ARGs located in phage particles, increasing the potential for ARGs transduction (Calero-Cáceres and Muniesa, 2016), which can be generalized, specialized or lateral (Chen et al., 2018; Penades et al., 2015; Thierauf et al., 2009). Phage particles carrying ARGs have been reported in sludge, soil, wastewater (Balcazar, 2014; Calero-Cáceres et al., 2014; Colomer-Lluch et al., 2014a, 2011b; Lekunberri et al., 2017; Ross and Topp, 2015), seawater (Calero-Cáceres and Balcázar, 2019), including marine ecosystems with a low anthropogenic impact (Blanco-Picazo et al., 2020), and in human and animal organisms (Brown-Jaque et al., 2018a, 2018b; Colomer-Lluch et al., 2011a). Recent studies have also confirmed that ARGs-carrying phage particles can end up in food products (vegetables, meat, and fish) apt for consumption, indicating that their presence in feces could be the result of phage mobilization by a circular oral-fecal-environmental-oral route (Blanco-Picazo et al., 2020; Gómez-Gómez et al., 2019; Larrañaga et al., 2018).

In this study, pasteurized milk and different pasteurized dairy products were analyzed for the presence of ARGs-carrying phages. This food group was selected as suitable for studying how antimicrobial resistance can be disseminated through closely linked routes involving animals, humans, and the environment, as outlined in the One-Health initiative (Hernando-Amado et al., 2019). Additionally, due to growing consumer demand for non-dairy plant milks, fresh horchata, a traditional Spanish beverage made from tiger nuts, was included in the study.

2. Material and methods

2.1. Samples

Samples of pasteurized milk (treated at 63 °C for 30 min), fermented lactic products (artisanal yogurt and kefir), fresh cheese prepared with pasteurized milk, and fresh, unpasteurized horchata, a nut-based milk, were analyzed. Ten samples of each were purchased from local retailers in the Barcelona area during 2020–2021. All samples were kept at 4 °C until analysis, which was performed within 24 h of purchase.

Twenty grams or milliliters of each sample were homogenized/mixed for 2 min in 60 ml of phage buffer (22 mM KH_2PO_4, 50 mM Na_2HPO_4, 85 mM NaCl, 1 mM $MgSO_4$, and 0.1 mM $CaCl_2$) using the Stomacher homogenizer (IUL Instruments GmbH, Königswinter, Germany). Stomacher bags with filters (Afora, Barcelona, Spain) were used to improve the separation of solid waste from the liquid fraction containing the microorganisms.

2.2. Bacterial and viral indicators

Serial decimal dilutions of the homogenates were used to evaluate in duplicate total aerobic microorganisms resistant to ampicillin, *E. coli* and ampicillin-resistant *E. coli*. Total aerobic bacteria were determined on Tryptone Soy Agar (Condalab, Madrid, Spain) with ampicillin (100 μg/ml) at 37 °C for 18 h. *E. coli* was determined on Chromocult® Coliform Agar (Merck, Darmstadt, Germany) without or with the addition of ampicillin (100 μg/ml) and incubated at 44 °C for 18 h. Suspected *E. coli* blue colonies obtained on Chromocult® Coliform Agar were confirmed with the Indole test and growth in McConkey Agar (BD, USA).

Somatic coliphages were determined in the samples for their role as fecal viral indicators (Jofre, 2007). Ten milliliters of the homogenates were centrifuged for 15 min at 4000 ×*g*, and the supernatant was filtered through 0.22 μm low protein binding polyethersulfone membranes (Millex-GP, Millipore, Bedford, MA). The filtrates were analyzed in duplicate for the presence of somatic coliphages according to the ISO standard method (Anonymous, 2000), which uses *E. coli* strain WG5 (ATCC 700078) as the bacterial host. Bacteriophage determination was performed by the double agar layer method and incubation at 37 °C for 18 h (Anonymous, 2000).

2.3. Purification of phage particles

As described in previous studies (Blanco-Picazo et al., 2020; Gómez-Gómez et al., 2019), the homogenate centrifuged at 4000 ×*g* and filtered through 0.22 μm polyethersulfone membranes was then treated with chloroform (1:10) (*v/v*) to remove the possible presence of vesicles containing DNA. Samples were vortexed for 5 min and centrifuged at 16,000 ×*g* for 5 min. The aqueous phase was separated and treated with DNase I (100 units/ml; Sigma-Aldrich, Spain) at 37 °C for 1 h to eliminate any free DNA present in the samples outside the phage particles. DNase was inactivated by heating for 5 min at 75 °C. After this step, aliquots were taken and used as controls in qPCR to rule out the presence of non-encapsidated DNA.

2.4. Phage DNA extraction

The phage suspensions were then processed to extract the DNA within the phage particles. Following treatment with proteinase K (20 mg/ml) in 250 μl of proteinase K buffer and incubation for 1 h at 55 °C (Sambrook and Russel, 2001), the encapsidated DNA was extracted by phenol-chloroform (1:1) (*v/v*) and chloroform treatment. DNA was precipitated using 100% ethanol and 3 M sodium acetate and resuspended in 50 μl of ultrapure water. DNA was quantified using a Qubit® Fluorometer (Life Technologies. CA. US).

2.5. Amplification of ARGs in DNA from phage particles

Quantitative real-time PCR (qPCR) using TaqMan hydrolysis probes was performed with a StepOne Real Time PCR System in a 20 μl reaction mixture with the TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA). The reaction contained 9 μl of the sample DNA or standards with known DNA concentration. The results were analyzed with the Applied Biosystems StepOne™ Instrument program. Eleven diverse ARGs with clinical relevance were evaluated: five genes that confer resistance to β-lactam antibiotics (bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CTX\text{-}M\text{-}9}$ group, $bla_{OXA\text{-}48}$ and bla_{VIM}); two quinolone resistance genes (*qnrA* and *qnrS*); a gene conferring resistance to methicillin (*mecA*), commonly found in *Staphylococcus* (Colomer-Lluch et al., 2011b); *armA*, which encodes aminoglycoside resistance and is widely distributed in *Enterobacteriaceae* (Galimand et al., 2005); and *sul1* and *tetW*, which confer resistance to sulfonamides and tetracycline, respectively, have wide application in the livestock industry and are frequently found in environmental bacterial populations (Cheng et al., 2013; Pruden et al., 2012).

For quantification, serial dilutions of a known concentration of gBlocks™ Gene Fragments (Integrated DNA Technologies, Coralville, IA, USA) for each ARG were used to generate the standard curves in each qPCR assay. All samples were run in triplicate (including the standards and negative controls). The number of gene copies (GC) was defined as the mean of the triplicate data obtained. To evaluate ARG abundance, the GC were calculated with the standard curves using the last valid cycle threshold for each ARG assay (Table 1), when the standard curve was consistent in the diverse replicates. The standards were also used as positive controls.

To ensure that only ARGs from the phage particles were detected, several controls were performed as previously described (Gómez-Gómez et al., 2019). Briefly, after filtration and treatment with chloroform and DNase and before proteinase K digestion for desencapsidation, an aliquot of each sample was tested to confirm the absence of each ARG and the bacterial *16S rRNA* by qPCR amplification. *16S rRNA* was amplified using the Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) (Table 1). Only samples negative for the respective ARGs and *16S rRNA* were considered free of non-encapsidated DNA and included in the analysis.

Table 1
Oligonucleotides used in this study

Target gene	Oligonucleotide	Sequence	Amplimer (bp)	LOQ (GC/µl)	Reference
bla_{TEM}	UP	CACTATTCTCAGAATGACTTGGT	85	35.2	(Lachmayr et al., 2009)
	LP	TGCATAATTCTCTTACTGTCATG			
	TaqMan TEM	6FAM-CCAGTCACAGAAAAGCATCTTACGG-MGBNFQ			
$bla_{CTX\text{-}M\text{-}1\text{-}}$ group	UP	ACCAACGATATCGCGGTGAT	101	37	(Colomer-Lluch et al., 2011b)
	LP	ACATCGCGACGGCTTTCT			
	TaqMan CTX-M-1	6FAM–TCGTGCGCCGCTG-MGBNFQ			
$bla_{CTX\text{-}M\text{-}9\text{-}}$ group	UP	ACCAATGATATTGCGGTGAT	85	35.4	(Colomer-Lluch et al., 2011a)
	LP	CTGCGTTCTGTTGCGGCT			
	TaqMan CTX-M-9	6FAM – TCGTGCGCCGCTG-MGBNFQ			
$bla_{OXA\text{-}48}$	UP	CGGTAGCAAAGGAATGGCAA	133	32.4	(Brown-Jaque et al., 2018a)
	LP	TGGTTCGCCCGTTTAAGATT			
	TaqMan OXA-48	6FAM-CGTAGTTGTGCTCTGGA-MGBNFQ			
bla_{VIM}	UP	AATGGTCTCATTGTCCGTGATG	61	33.9	(Larrañaga et al., 2018)
	LP	TCGCACCCCACGCTGTA			
	TaqMan VIM	6FAM–TGATGAGTTGCTTTTGATTG-MGBNFQ			
sul1	UP	CCGTTGGCCTTCCTGTAAAG	67	34.8	(Calero-Cáceres et al., 2014)
	LP	TTGCCGATCGCGTGAAGT			
	TaqMan sul1	6FAM-CGAGCCTTGCGGCGG-MGBNFQ			
mecA	UP	CGCAACGTTCAATTTAATTTTGTTAA	92	33.8	(Volkmann et al., 2004)
	LP	TGGTCTTTCTGCATTCCTGGA			
	TaqMan mecA	6FAM-AATGACGCTATGATCCCAATCTAACTTCCACA-MGBNFQ			
qnrA	UP	AGGATTGCAGTTTCATTGAAAGC	138	31.1	(Colomer-Lluch et al., 2014b)
	LP	TGAACTCTATGCCAAAGCAGTTG			
	TaqMan qnrA	6FAM-TATGCCGATCTGCGCGA-MGBNFQ			
qnrS	UP	CGACGTGCTAACTTGCGTGA	118	34.6	(Colomer-Lluch et al., 2014b)
	LP	GGCATTGTTGGAAACTTGCA			
	TaqMan qnrS	6FAM-AGTTCATTGAACAGGGTGA-MGBNFQ			
armA	UP	GAAAGAGTCGCAACATTAAATGACTT	94	33.4	(Quirós et al., 2014)
	LP	GATTGAAGCCACAACCAAAATCT			
	TaqMan armA	6FAM-TCAAACATGTCTCATCTATT-MGBNFQ			
tetW	UP	GACGGACACCATGTTTTTGGA	62	34.8	(Blanco-Picazo et al., 2020)
	LP	AGGAAGTGACTGCCGCTTGA			
	TaqMan tetW	6FAM-AGCGTGGGATTACCA-MGBNFQ			
16SrRNA	338F	ACTCCTACGGGAGGCAGCAG	236		(Weisburg et al., 1991)
	518R	ATTACCGCGGCTGCTGG			

2.6. Propagation cultures

The ability of phage particles in the samples to infect a host strain and propagate was evaluated. The strain *E. coli* WG5, also used to enumerate somatic coliphages, was selected because of its susceptibility to phage infection (Larrañaga et al., 2018), and because its genome does not contain any of the ARGs targeted in this study or any prophage (Imamovic et al., 2018).

Propagation cultures were prepared with 1 ml of the phage suspension (obtained after filtration, chloroform and DNAse treatment) and 1 ml of *E. coli* WG5 in the exponential phase (OD_{600} 0.3) in 8 ml of Luria-Bertani broth (LB) and then incubated overnight at 37 °C with shaking. After incubation, the phages were purified by filtration through 0.22 µm low protein binding polyethersulfone membranes and treated with chloroform and DNase as indicated above.

The infectivity of the phage particles carrying ARGs was evaluated by comparing the abundance of ARGs by qPCR before (direct quantification from the sample) and after phage propagation in the enrichment cultures. If the ARGs copy numbers in phage particles increased after propagation, it was considered that the phage particles were able to infect and propagate on the host strain. Otherwise, if ARGs levels decreased, the particles were considered incapable of propagating.

2.7. Transmission electron microscopy

The presence of phage particles in the supernatant of enrichment cultures after the propagation step was verified by observation with a transmission electron microscope (TEM). Phages were purified as described above from 2 ml of the supernatant and 100× concentrated using protein concentrators (100-kDa Amicon Ultra centrifugal filter units; Millipore, Bedford, MA) with gentle centrifugation at 6000 ×*g* for the time necessary to reduce the volume to 20 µl. Seven µl of the concentrated phage suspension was dropped onto copper grids with carbon-coated Formvar films and negatively stained with 2% ammonium molybdate (pH 6.8) for 1.5 min. Phages were visualized using a Jeol 1010 TEM (JEOL Inc. Peabody, MA USA) operating at 80 kV.

3. Results and discussion

3.1. Microbiological parameters

Microbiological criteria are used to ensure safety in food products and as a marker of good hygiene in the manufacturing process. The levels of microbial indicators observed in the dairy samples in this study were mostly within the limits established by EU regulations for safe consumption (Commission Regulation EC No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs, 2005).

Aerobic ampicillin-resistant bacteria were detected in all samples analyzed except in yogurt (Table 2), with average values especially high in fresh cheese and horchata (close to 10^9 cfu/25 g or cfu/100 ml sample, respectively). *E. coli*, used as a bacterial indicator of process hygiene, was detected in kefir, fresh cheese and horchata (Table 2). The highest average values of *E. coli* were found in fresh cheese, with one sample deemed unfit for consumption, as it failed to meet the EU requirements for this parameter (Commission Regulation EC No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs, 2005). This fresh cheese sample also contained ampicillin-resistant *E. coli*, which was targeted in the analysis as this resistance is common in the most frequently used indicator bacteria (The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2018/2019, 2021). Predictably, the highest values of ampicillin-resistant *E. coli* were obtained in fresh cheese and horchata, the abundance being similar to that of *E. coli*. No bacterial indicators were observed in yogurt samples, which is

Table 2
Bacterial (CFU/25 g) and viral (PFU/25 g) indicators in dairy products and a plant-based milk.

Microorganism		Milk[a]	Kefir	Fresh cheese	Yogurt	Horchata[a]
n		10	10	10	10	10
Total aerobic bacteria amp^R	%	40	50	60	0	70
	Average	7.1×10^6	1.3×10^6	1.9×10^9	0	1.4×10^9
	SD	8.1×10^6	2.0×10^6	3.1×10^9	0	3.7×10^9
E. coli	%[b]	0	20	20	0	3.0×10^1
	Average	0	8.2×10^4	3.9×10^6	0	4.1×10^4
	SD	0	1.2×10^5	5.5×10^6	0	6.9×10^4
E. coli amp^R	%[b]	0	0	10	0	0
	Average	0	0	1.7×10^3	0	0
	SD	0	0	–	0	0
Somatic coliphages	%[b]	0	10	20	10	30
	Average	0	7.5×10^1	6.5×10^3	7.5×10^0	8.0×10^2
	SD	–	–	1.9×10^3	–	8.7×10^2

[a] CFU/100 ml; PFU/100 ml.
[b] Percentage of positive samples.

in accordance with the pasteurization or ultrapasteurization process to which raw milk is subjected before yogurt manufacture (Aryana and Olson, 2017).

The analysis also included somatic coliphages, as they have been proposed as fecal viral indicators and their presence is indicative of fecal pollution and the potential presence of viruses of fecal origin. All product types were found to contain somatic coliphages except for milk, where the pasteurization process may have inactivated infectious phages at least below the limit of detection of the double agar layer technique used. Somatic coliphages have recently been included in drinking water management policies and new EU directives for drinking and reused water (Council of the European Union, 2020; WHO, 2017), but to date they have not been incorporated in food safety policies. Therefore, there is no established range of somatic coliphage values that correlate with fecal pollution and suitability for consumption.

3.2. Detection of ARGs in phage DNA fraction

ARGs were detected in phage DNA in all sampled foodstuffs, either directly or after propagation in *E. coli*. Depending on the ARG and the product, 10–100% of samples tested positive, kefir being the matrix where all targeted ARGs were detected, some only after propagation (Fig. 1). Despite the heterogeneity of the results, β-lactamase genes and *sul1* were the predominant ARGs in all the samples. Among β-lactamase genes, bla_{TEM} was the most prevalent, found in 90–100% of samples, followed by $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$, the latter detected in 90% of milk samples by direct analysis (Fig. 1). Propagation resulted in an increase in samples positive for bla_{VIM} in all products except yogurt, and $bla_{OXA\text{-}48}$ in milk, fresh cheese and horchata. Regarding *sul1*, 80% of kefir, fresh cheese and horchata samples were positive by direct analysis. A high prevalence of *armA* and *tetW* was also found in all the matrices; in particular, 70% of milk samples were positive for both. After propagation, *mecA* was detected in milk, kefir and fresh cheese (Fig. 1), and *qnrA* only in one sample of kefir, which also contained *mecA* and *qnrS*.

3.3. Propagation of phage particles containing ARGs

The ARG counts in phage DNA after propagation on *E. coli* WG5 were compared with those observed by direct analysis (Fig. 2). Propagation of phage suspensions revealed ARGs in samples where the ARG copy number was below the detection limit when analyzed directly. Moreover, an increase in ARG copy numbers after the enrichment step indicated that at least some of the ARG-carrying phage particles were able to

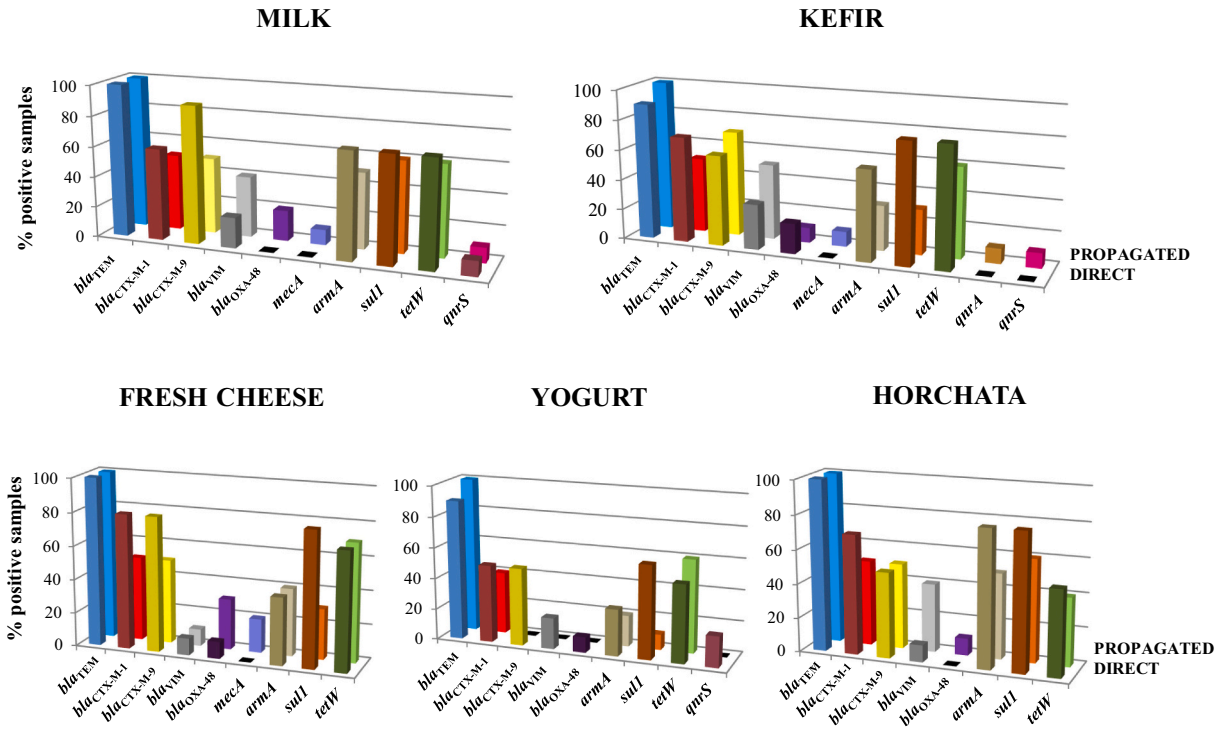

Fig. 1. Percentage of samples of each matrix (milk, fresh cheese, kefir, yogurt and horchata) where ARGs were detected in the DNA extracted from the viral fraction.

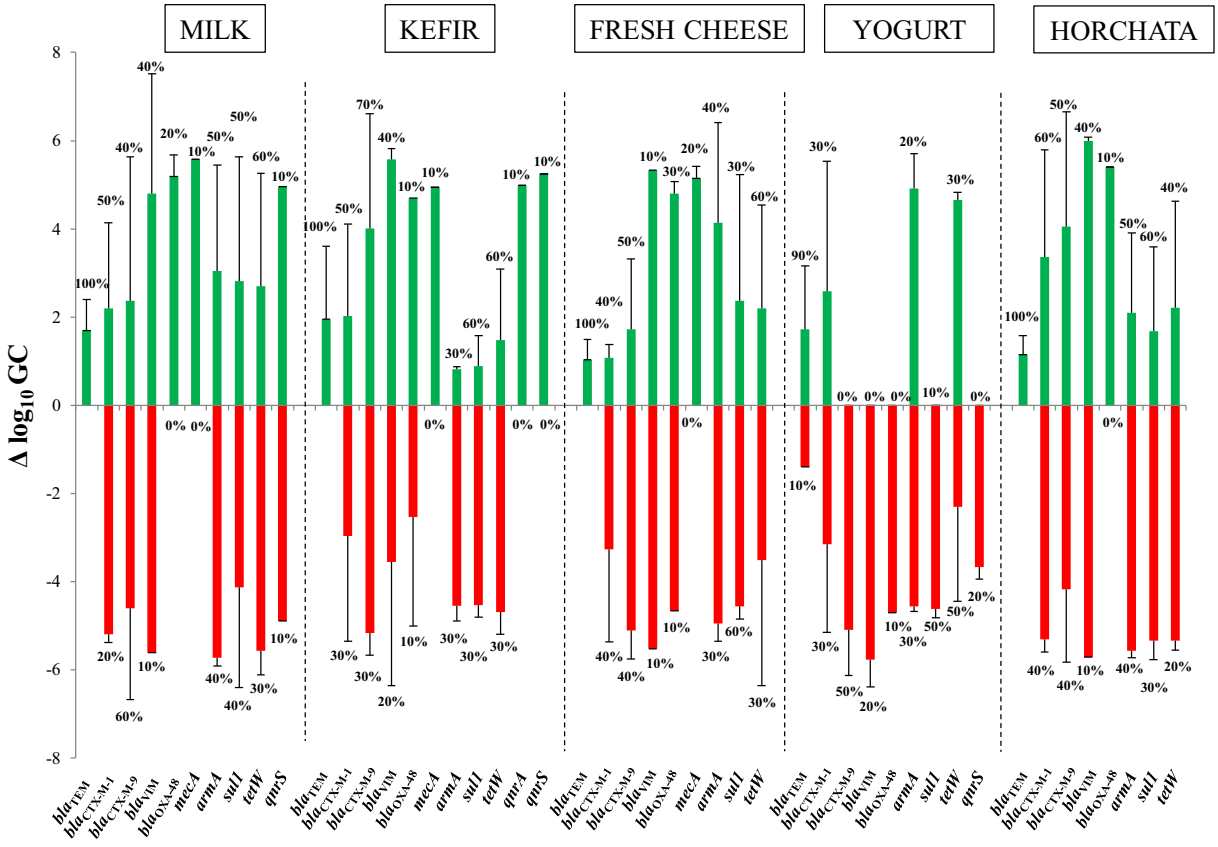

Fig. 2. Logarithmic variation in gene copy (GC) numbers of each ARG detected in the viral DNA fraction of milk, kefir, fresh cheese, yogurt and horchata samples after the propagation step. Results are grouped by the sample type. Green bars in the upper chart show the ARGs in each matrix with higher GC values after propagation. Red bars show the ARGs in each matrix with lower GC values after propagation. Bars are the average $\log_{10}$ GC variation in the samples of each matrix and for each ARG. At the top of each bar is indicated the percentage of samples of each matrix with an increase or reduction in the $\log_{10}$ GC values.

infect an *E. coli* strain and propagate (Fig. 2, green columns), providing information about their infectious nature. In these cases, phages containing the ARG may have incorporated the gene in the phage genome by mechanisms similar to specialized transduction (Schneider, 2017), which generates phages with a functional genome able to initiate a lytic cycle. In contrast, when ARGs were not detected or the copy numbers decreased (Fig. 2 red columns), phage particles were considered non-infectious, either because the *E. coli* WG5 strain was not a suitable host, the particles were damaged, or because the ARGs were inside transducing particles containing only bacterial DNA and consequently unable to propagate as a phage. These transducing particles are then suspected to be derived from generalized or lateral transduction events (Chen et al., 2018; Thierauf et al., 2009).

After propagation, the copy numbers of practically all targeted ARGs increased in all the matrices except yogurt, where the reductions of 1.3–5.7 $\log_{10}$ GC (Fig. 2) were in agreement with the lower number of phages detected in the samples and in the previous experiments. It should be noted that when there is a reduction in ARG copies, the decrease of $\log_{10}$ GC units does not correspond only to the dilution factor (which should be 1/10), and sometimes the decrease was higher. It can be due to other factors associated with the propagation of the phages and structural stability of the particles. These include particle damage, injection of nonspecific DNA, an impaired recovery from the enrichment culture due to abundant cellular debris, or values close to or above the qPCR limit of quantification, which hinders accurate quantification.

Comparing the effect of phage propagation on the different ARGs (Fig. S1), the highest increase in GC number was observed in the β-lactamase genes bla_{VIM} and $bla_{\text{oxa-48}}$, which on average increased by more than 5 $\log_{10}$ GC. The most prevalent ARG, bla_{TEM}, increased by almost 2 $\log_{10}$ GC after propagation in all samples, except one of yogurt, whereas *mecA* and *qnrA* genes increased by up to 5 $\log_{10}$ GC in all the samples where they were present (Fig. 2). One of the least prevalent ARGs, *tetW* presented the highest percentage of samples with a higher GC after propagation (30%–70% of positive samples). There is no explanation for the different propagation levels of the phage particles containing different ARGs. Some ARGs might be more prone to be packaged because of specialized transduction events due to their genomic location in the original host, hence, generating infectious particles. In contrast, other ARGs might be located downstream prophages, being more frequently mobilized by lateral transduction (Chen et al., 2018) in transducing particles unable to propagate.

It should be mentioned that the propagation experiments were carried out on *E. coli* WG5 because this host strain is highly sensitive for the detection of phages of fecal origin, some of which might harbor ARGs. It was also chosen because its genome does not contain prophages or ARGs (Imamovic et al., 2018), ensuring that the phages detected after propagation originated from the sample. Although these particular traits made it a useful tool for this study, strain WG5 is not necessarily the best host for the enrichment of phages from dairy samples or horchata, and the use of dairy-associated LAB or bacteria found in plants would likely reveal the presence of other infectious ARGs-carrying phages.

3.4. Phage particle visualization after enrichment

TEM visualization of phage particles required a minimal titer (10^8 particles), which in the case of milk and yogurt (previously found to contain few or no phages) was not achieved even after successive concentration steps. In the case of fresh cheese, kefir and horchata, phage capsids were observed. Three different phage morphological types were visualized: in fresh cheese some with capsid diameters of 70 nm and

contractile tails, corresponding to the *Myoviridae* type were observed (Fig. 3A). In horchata phages with icosahedral capsid of 50 nm and non-contractile tails (Fig. 3B) and in kefir, phage particles with elongated capsids of 50 nm long and 38 nm width and non-contractile tails (Fig. 3C and D), both corresponding to the *Siphoviridae* morphological types (International Committee on Taxonomy of Viruses, 2011), were visualized.

3.5. Origin of the ARG-containing phage particles

The presence of phages in dairy products is not unexpected, given their ubiquity. Phage particles, and the DNA they package, can persist outside bacteria and endure for longer than the host strain (Calero-Cáceres and Muniesa, 2016). The origin of phage particles in the analyzed samples could be the raw material (raw milk or nuts in the case of horchata) (Madera et al., 2004), the starter strains (Chopin et al., 2001) used in some of the products, although phages originating from the latter should not be able to propagate in *E. coli*, or contamination during the production process (Marcin, 2020). Milk can contain bacteria from different sources (Quigley et al., 2013) and free phage virions can also reach milk due to their unique capacity to bypass anatomical and physiological barriers (Dinleyici et al., 2021; Huh et al., 2019). Phages can also be induced from milk microbiota carrying prophages (Garneau and Moineau, 2011), which sometimes interferes with fermentation processes. In any case, regardless their origin, phages can remain in milk even after pasteurization process (Atamer et al., 2013). The ingestion of phages in dairy products should be innocuous for the consumer, considering they are present in our intestinal microbiota (Fernández-Orth et al., 2019; Reyes et al., 2010) as well as in other human and animal biomes (Letarov and Kulikov, 2009; Navarro and Muniesa, 2017; Nguyen et al., 2017).

3.6. Impact on food industry

As phages can cause starter-culture failure by attacking LAB, they have been traditionally regarded as a contaminant and source of financial loss in the dairy product industry (Pujato et al., 2019). However, new beneficial applications for phages have been proposed for the enhancement of safety in food production (García et al., 2008; O'Sullivan et al., 2019). There is growing interest in phages as an ecological alternative to antibiotics and chemical products used as preservatives and surface disinfectants along the food production chain (Campbell, 2003). Certain phage cocktails are recognized as safe for biocontrol by the Food and Drug Administration (US-FDA) (Kawacka et al., 2020), and phages have been granted a qualified presumption of safety by the European Food Safety Authority (EFSA) (Kawacka et al., 2020). Thus, phages have a range of potential applications in the food industry, including pathogen elimination (Schmelcher and Loessner, 2014), removal of biofilms (Gutiérrez et al., 2016) and the treatment of surfaces and installations (O'Sullivan et al., 2019).

There is still another way that phages can impact food and the environment, which has received less attention: their role as vehicles of horizontal gene transfer, particularly in the dissemination of ARGs (Koutsoumanis et al., 2021; Lood et al., 2017). Besides certain fecal bacteria, phages are included in regulations for microorganism control in food, but only for their interference with fermentation processes, and not as a potential reservoir of genes related with virulence. The reporting of phages or phage particles containing ARGs in different food types (Blanco-Picazo et al., 2020; Gómez-Gómez et al., 2019; Jebri et al., 2021; Larrañaga et al., 2018) and related environments (Anand et al., 2016; Colombo et al., 2016; Koutsoumanis et al., 2021; Ross and Topp, 2015; Wang et al., 2018) has increased awareness that their monitoring is advisable.

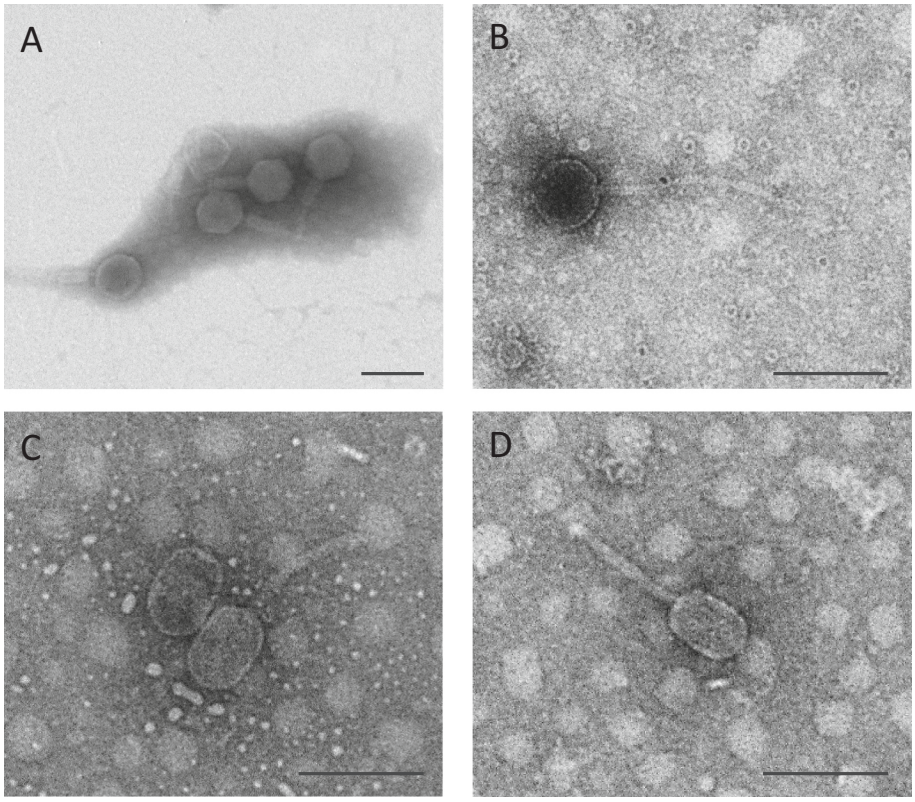

Fig. 3. Phage particle visualization by transmission electron microscopy after negatively stain with 2% ammonium molybdate. Phages were observed from fresh cheese (A), horchata (B) and kefir (C and D). Bar 100 nm.

4. Conclusion

In this study, at least a fraction of the phage population in the food samples was found to be infective which was correlated with their capacity to increase the number of ARG copies, which in turn would be released in high numbers. Moreover, both transducing particles and complete phages can transfer ARGs to suitable recipient bacteria, thereby enhancing the chances of generating new resistant strains. These events can take place in the food matrix (Colavecchio et al., 2017), during dairy food production (Picozzi et al., 2012) or even in the intestinal tract after ingestion (Colavecchio et al., 2017). In this regard, the absence of policies addressing the occurrence of ARGs-carrying phage particles in food represents a gap in the stewardship of antibiotic resistance dissemination as considered by the WHO (2018).

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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.

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