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Antimicrobial activity, antibiotic susceptibility and virulence factors of Lactic Acid Bacteria of aquatic origin intended for use as probiotics in aquaculture

Estefanía Muñoz-Atienza[1], Beatriz Gómez-Sala[1], Carlos Araújo[1,2], Cristina Campanero[1], Rosa del Campo[3], Pablo E Hernández[1], Carmen Herranz[1] and Luis M Cintas[1*]

Abstract

Background: The microorganisms intended for use as probiotics in aquaculture should exert antimicrobial activity and be regarded as safe not only for the aquatic hosts but also for their surrounding environments and humans. The objective of this work was to investigate the antimicrobial/bacteriocin activity against fish pathogens, the antibiotic susceptibility, and the prevalence of virulence factors and detrimental enzymatic activities in 99 Lactic Acid Bacteria (LAB) (59 enterococci and 40 non-enterococci) isolated from aquatic animals regarded as human food.

Results: These LAB displayed a broad antimicrobial/bacteriocin activity against the main Gram-positive and Gram-negative fish pathogens. However, particular safety concerns based on antibiotic resistance and virulence factors were identified in the genus *Enterococcus* (86%) (*Enterococcus faecalis*, 100%; *E. faecium,* 79%). Antibiotic resistance was also found in the genera *Weissella* (60%), *Pediococcus* (44%), *Lactobacillus* (33%), but not in leuconostocs and lactococci. Antibiotic resistance genes were found in 7.5% of the non-enterococci, including the genera *Pediococcus* (12.5%) and *Weissella* (6.7%). One strain of both *Pediococcus pentosaceus* and *Weissella cibaria* carried the erythromycin resistance gene *mef*(A/E), and another two *P. pentosaceus* strains harboured *lnu*(A) conferring resistance to lincosamides. Gelatinase activity was found in *E. faecalis* and *E. faecium* (71 and 11%, respectively), while a low number of *E. faecalis* (5%) and none *E. faecium* exerted hemolytic activity. None enterococci and non-enterococci showed bile deconjugation and mucin degradation abilities, or other detrimental enzymatic activities.

Conclusions: To our knowledge, this is the first description of *mef*(A/E) in the genera *Pediococcus* and *Weissella*, and *lnu*(A) in the genus *Pediococcus*. The *in vitro* subtractive screening presented in this work constitutes a valuable strategy for the large-scale preliminary selection of putatively safe LAB intended for use as probiotics in aquaculture.

Keywords: Lactic Acid Bacteria, Aquatic animals, Aquaculture probiotics, Anti-fish pathogens activity, Antibiotic resistance and virulence factors, Qualified Presumption of Safety

Background

Aquaculture has the potential to make a significant contribution to the increasing demand for aquatic food in most world regions; however, in order to achieve this goal, the sector will have to face significant challenges, including the production intensification, the disease control and the prevention of the environmental deterioration [1]. In fish farming, the widespread use of antibiotics as prophylactic and therapeutic agents to control bacterial diseases has been associated with the emergence of antibiotic resistance in bacterial pathogens and with the alteration of the microbiota of the aquaculture environment [2,3]. This resulted in the ban of antibiotic usage as animal growth promoters in Europe and stringent worldwide regulations on therapeutical antibiotic applications. This scenario has led to an evergrowing interest in the search and

* Correspondence: lcintas@vet.ucm.es
[1]Grupo de Seguridad y Calidad de los Alimentos por Bacterias Lácticas, Bacteriocinas y Probióticos (Grupo SEGABALBP) Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid 28040, Spain
Full list of author information is available at the end of the article

development of alternative strategies for disease control, within the frame of good husbandry practices, including adequate hygiene conditions, vaccination programmes and the use of probiotics, prebiotics and immunostimulants [4-6]. Recently, novel strategies to control bacterial infections in aquaculture have emerged, such as specific killing of pathogenic bacteria by bacteriophages, growth inhibition of pathogen by short-chain fatty acids and polyhydroxyalkanoates, and interference with the regulation of virulence genes (quorum sensing disruption), which have been reviewed by Defoirdt *et al.* [7]. With regard to probiotics, they are defined as live microbial adjuncts which have a beneficial effect on the host by: (i) modifying the host-associated or ambient microbial community; (ii) improving feed use or enhancing its nutritional value; (iii) enhancing the host response towards disease; and/or (iv) improving its environment [8]. To date, most probiotics proposed as biocontrollers and bioremediation agents for aquaculture belong to the LAB group (mainly to the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus* and *Carnobacterium*), to the genera *Vibrio*, *Bacillus*, and *Pseudomonas* or to the species *Saccharomyces cerevisiae* [8,9]. Recently, a probiotic culture (Bactocell®, *Pediococcus acidilactici* CNCM MA18/5 M) has been authorized for the first time for use in aquaculture in the European Union.

According to the FAO/WHO [10], the development of commercial probiotics requires their unequivocal taxonomic identification, as well as their *in vitro* and *in vivo* functional characterization and safety assessment. In Europe, the European Food Safety Agency (EFSA) proposed a system for a pre-market safety assessment of selected groups of microorganisms used in food/feed and the production of food/feed additives leading to a Qualified Presumption of Safety (QPS) status [11-13]. The QPS approach propose that the safety assessment of a defined taxonomic group could be made based on establishing taxonomic identity, body of knowledge, possible pathogenicity and commercial end use. According to the EFSA approach [13], most LAB species are included in the QPS list and, therefore, demonstration of their safety only requires confirmation of the absence of determinants of resistance to antibiotics of human and veterinary clinical significance. However, in the case of enterococci, a more thorough, strain-specific evaluation is required to assess the risk associated to their intentional use in the food chain. In this work, we present the antimicrobial activity against fish pathogens and the *in vitro* safety assessment beyond the QPS approach of a collection of 99 LAB belonging to the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Weissella*, previously isolated from aquatic animals regarded as human food [14] and intended for use as probiotics in aquaculture.

Results

Direct antimicrobial activity of the 99 LAB of aquatic origin

The 99 LAB strains isolated from fish, seafood and fish products displayed direct antimicrobial activity against, at least, four of the eight tested indicator microorganisms (Table 1). The most sensitive indicators were *Listonella anguillarum* CECT4344, *Ls. anguillarum* CECT7199 and *Aeromonas hydrophila* CECT5734, followed by *Lactococcus garvieae* JIP29-99, *Streptococcus iniae* LMG14521 and *Streptococcus agalactiae* CF01173. On the contrary, *Photobacterium damselae* CECT626 and *Vibrio alginolyticus* CECT521 were the less sensitive indicator microorganisms.

Preliminary safety evaluation of enterococci: presence of virulence factors, production of gelatinase and hemolysin and antibiotic susceptibility

Concerning *E. faecalis*, most of the strains (20 strains, 95%) harboured, at least, one relevant virulence factor: *efaAfs* (95%), *gelE* (71%), or *agg* (67%) genes (Table 2). A positive gelatinase reaction was found in 15 *E. faecalis* strains (71%) which harboured *gelE*, from which 12 also harboured *agg* gene. Only one *E. faecalis* strain (*E. faecalis* SDP10) (5%), harbouring $cylL_L$-$cylL_S$-*cylM*, exerted hemolytic activity, while none of the strains amplified *hyl* or *esp* genes. With regard to *E. faecium*, 20 strains (53%) harboured, at least, one relevant virulence factor: *efaAfs* (45%), *gelE* (24%) or *agg* (8%), but only 4 strains (11%) exerted gelatinase activity. None of the *E. faecium* strains exerted hemolytic activity nor amplified *hyl* or *esp* genes. The results of the antibiotic susceptibility tests revealed that 39 enterococccal strains (66%) displayed acquired antibiotic resistance to antibiotics other than penicillin G, chloramphenicol and high-level gentamicin. In this respect, 13 *E. faecalis* strains (62%) showed acquired resistance to (i) second generation quinolones (ciprofloxacin and/or norfloxacin) (12 strains, 57%), (ii) rifampicin (5 strains, 24%), (iii) nitrofurantoin (5 strains, 24%), (iv) glycopeptides (vancomycin and teicoplanin) (4 strains, 19%), and/or (v) erythromycin (1 strain, 5%). However, 26 *E. faecium* strains (68%), including 17 strains that encode virulence factors and nine strains without these traits, displayed acquired resistance to (i) erythromycin (14 strains, 37%), (ii) nitrofurantoin (11 strains, 29%), (iii) second generation quinolones (ciprofloxacin and/or norfloxacin) (10 strains, 26%), (iv) rifampicin (4 strains, 11%), (v) tetracycline (2 strains, 5%), and/or (vi) glycopeptides (vancomycin and teicoplanin) (1 strain, 3%). Moreover, multiple antibiotic resistance (two to six antibiotics) was found in *E. faecalis* (10 strains, 48%) and, to a lesser extent, in *E. faecium* (12 strains, 32%) (Table 2). According to the results above, 21 *E. faecalis* strains were discarded for further studies based on the presence of virulence factors (8 strains,

Table 1 Origin and direct antimicrobial activity against fish pathogens of LAB isolated from aquatic animals

Origin	Strain		Indicator microorganisms[a]							
			Lactococcus garvieae JIP29-99	*Streptococcus agalactiae* CF01173	*Streptococcus iniae* LMG14521	*Aeromonas hydrophila* CECT5734	*Listonella anguillarum* CECT4344	*Ls. anguillarum* CECT7199	*Photobacterium damselae* CECT626	*Vibrio alginolyticus* CECT521
Albacore (*Thunnus alalunga*)	*Enterococcus faecium*	BNM58	+	+	+	++	++	+++	+	-
	Weissella cibaria	BNM69	+	+	+	+++	+++	+++	-	-
Atlantic salmon (*Salmo salar*)	*Enterococcus faecalis*	SMF10	+	+	+	++	+++	++	-	+
		SMF28	+	+	++	++	+++	+	-	+
		SMF37	+	+	+	+	++	+++	-	+
		SMF69	+	+	++	++	+++	+++	+	+
		SMM67	+	+	++	++	+++	+++	-	-
		SMM70	+	+	+	+	+++	+++	-	-
	E. faecium	SMA1	+	+	+	++	++	+++	+	-
		SMA7	+	+	+	+	++	+++	+	+
		SMA8	+	+	+	++	++	+++	+	+
		SMA101	+	+	+	++	+++	++	+	+
		SMA102	+	+	+	++	+++	+	+	+
		SMA310	++	+	+	++	+++	++	+	+
		SMA320	++	+	+	++	++	+++	+	+
		SMA361	+	+	+	++	++	+++	+	+
		SMA362	+	+	+	++	++	+++	+	-
		SMA384	+	+	+	++	++	+++	+	-
		SMA389	+	+	+	++	++	+++	-	+
		SMF8	+	+	++	++	++	++	+	-
		SMF39	+	+	++	++	++	+++	+	+
	Lactobacillus sakei subsp. *carnosus* (*Lb. carnosus*)	SMA17	+	-	+	++	+++	+++	-	-
	Lactococcus lactis subsp. *cremoris* (*L. cremoris*)	SMF110	+	+	+	+	+++	+++	+	+
		SMF161	+	+	+	++	+++	+++	+	++
		SMF166	+	+	+	++	++	+++	+	++
	Leuconostoc mesenteroides subsp. *cremoris* (*Lc. cremoris*)	SMM69	+	+	+	++	+++	+++	-	-

Table 1 Origin and direct antimicrobial activity against fish pathogens of LAB isolated from aquatic animals *(Continued)*

	Pediococcus pentosaceus	SMF120	++	++	++	++	+++	+++	-	+
		SMF130	++	+	++	++	+++	+++	-	+
		SMM73	++	+	+	+++	+++	+++	+	++
	W. cibaria	SMA14	++	+	+	++	+++	+++	+	++
		SMA25	+	+	+	+++	+++	+++	-	-
Cod (*Gadus morhua*)	*E. faecalis*	BCS27	++	++	++	++	+++	+++	-	-
		BCS32	+	+	+	+	++	+++	-	+
		BCS53	+	++	+	+	+++	+++	+	-
		BCS67	+	+	-	++	+++	++	-	+
		BCS72	+	+	+	++	+++	+++	+	-
		BCS92	+	+	+	++	+++	++	+	+
	E. faecium	BCS59	++	+	++	++	+++	+++	-	+
		BCS971	+	+	+	+	+++	+++	-	+
		BCS972	+	+	+	+	+++	+++	-	+
	Lactobacillus curvatus subsp. *curvatus* (*Lb. curvatus*)	BCS35	-	-	+	++	+++	+++	-	-
	Lc. cremoris	BCS251	+	+	++	+	+++	+++	-	+
		BCS252	+	+	++	+	+++	+++	-	+
	P. pentosaceus	BCS46	++	+	++	+++	+++	+++	-	+
	W. cibaria	BCS50	++	+	++	++	+++	+++	-	+
Common cockle (*Cerastoderma edule*)	*E. faecium*	B13	+	+	++	++	+++	+++	-	-
		B27	+	+	+	++	+++	++	+	+
	Lb. carnosus	B43	+	+	+	++	+++	+++	-	-
	P. pentosaceus	B5	++	+	++	++	+++	+++	-	-
		B11	++	+	++	+++	+++	+++	+	-
		B41	++	++	++	+++	+++	+++	+	++
		B260	++	+	++	++	+++	+++	-	++
	W. cibaria	B4620	++	+	++	++	+++	+++	-	++
Common ling (*Molva molva*)	*E. faecium*	MV5	+	+	+	++	++	+++	+	+
Common octopus (*Octopus vulgaris*)	*E. faecalis*	P77	++	+	++	++	+++	+++	-	+

Table 1 Origin and direct antimicrobial activity against fish pathogens of LAB isolated from aquatic animals *(Continued)*

	E. faecium	P68	++	+	+++	++	+++	+++	-	+
		P623	+	+	+	+	+++	++	-	+
	P. pentosaceus	P63	++	+	++	+++	+++	+++	-	+
		P621	++	+	++	+	+++	+++	-	+
	W. cibaria	P38	++	++	++	++	+++	+++	-	+
		P50	++	+	+	++	+++	+++	-	+
		P61	++	+	+	++	+++	+++	-	-
		P64	++	+	+	+++	+++	+++	+	++
		P69	++	+	+	++	+++	+++	+	++
		P71	+	+	++	++	+++	+++	+	+
		P73	++	++	++	++	+++	+++	-	+
		P622	++	++	++	+	+++	+++	+	+
European seabass (*Dicentrarchus labrax*)	*E. faecium*	LPP29	+	+	+	+	++	+++	+	-
	P. pentosaceus	LPM78	++	+	++	++	+++	+++	-	-
		LPM83	++	+	++	++	+++	+++	-	-
		LPP32	++	++	++	++	+++	+++	-	+
		LPV46	++	+	++	++	+++	+++	-	+
		LPV57	++	+	++	+++	+++	+++	-	-
European squid (*Loligo vulgaris*)	*E. faecium*	CV1	+	+	+	+	+++	+++	-	+
		CV2	++	+	+	+	+++	++	+	+
Megrim (*Lepidorhombus boscii*)	*E. faecalis*	GM22	-	-	+	++	++	+++	+	++
		GM26	-	-	+	+	++	++	+	-
		GM33	-	-	++	+	++	+++	+	-
	E. faecium	GM23	+	+	+	++	++	+++	+	+
		GM29	++	++	+	++	++	+++	+	+
		GM351	-	-	+	+	++	++	+	-
		GM352	++	+	+	++	++	+++	+	+
Norway lobster (*Nephrops norvegicus*)	*E. faecalis*	CGM16	++	+	++	++	+++	+++	-	+
		CGM156	+	+	++	++	+++	+++	-	-

Table 1 Origin and direct antimicrobial activity against fish pathogens of LAB isolated from aquatic animals *(Continued)*

		CGM1514	+	+	+	++	+++	++	+	+
		CGV67	++	+	+	+	+++	+++	+	+
	E. faecium	CGM171	+	+	+	+	+++	+++	+	+
		CGM172	+	+	+	+	+++	+++	+	+
Rainbow trout (*Oncorhynchus mykiss*)	*E. faecium*	TPM76	+	+	+	+	++	+++	+	+
		TPP2	+	+	+	+	++	+++	+	+
	P. pentosaceus	TPP3	++	+	+	++	+++	+++	-	++
Sardine (*Sardina pilchardus*)	*E. faecalis*	SDP10	+	+	+	+	+++	+++	-	+
	W. cibaria	SDM381	++	+	++	++	+++	+++	-	-
		SDM389	+	+	++	++	+++	+++	-	-
Swimcrab (*Necora puber*)	*E. faecium*	NV50	+	+	+	++	++	++	+	-
		NV51	++	+	+	+	++	++	+	++
		NV52	++	+	+	+	++	+++	+	+
		NV54	++	+	+	+	++	+++	+	+
		NV56	++	+	+	++	++	++	+	-

[a]Direct antimicrobial activity was determined by a SOAT and the scores reflect different degrees of growth inhibition (diameter in mm); -, no inhibition; +, 3–5 mm inhibition zone; ++, 6–9 mm inhibition zone; +++, ≥10 mm inhibition zone.

Table 2 Preliminary safety evaluation of enterococci

Enterococcus spp.	Strain	Virulence Factors		Antibiotic resistance phenotype[c]
		Genotype[a]	Phenotype[b]	
E. faecalis	SMF10	*efaAfs*$^+$, *gelE*$^+$, *agg*$^+$	GelE$^+$, Hly$^-$	CIP, NOR
	SMF28	*efaAfs*$^+$, *gelE*$^+$	GelE$^+$, Hly$^-$	CIP, NOR
	SMF37	*efaAfs*$^+$, *gelE*$^+$, *agg*$^+$	GelE$^+$, Hly$^-$	-
	SMF69	*efaAfs*$^+$, *gelE*$^+$, *agg*$^+$	GelE$^+$, Hly$^-$	CIP, RIF
	SMM67	n.d.	GelE$^-$, Hly$^-$	CIP, NIT, NOR, TEC, VAN
	SMM70	*efaAfs*$^+$, *gelE*$^+$	GelE$^+$, Hly$^-$	ERY, NIT
	BCS27	*efaAfs*$^+$, *gelE*$^+$, *agg*$^+$	GelE$^+$, Hly$^-$	CIP, NIT, NOR, RIF, TEC, VAN
	BCS32	*efaAfs*$^+$, *gelE*$^+$, *agg*$^+$	GelE$^+$, Hly$^-$	NOR
	BCS53	*efaAfs*$^+$, *gelE*$^+$, *agg*$^+$	GelE$^+$, Hly$^-$	-
	BCS67	*efaAfs*$^+$	GelE$^-$, Hly$^-$	CIP
	BCS72	*efaAfs*$^+$, *agg*$^+$	GelE$^-$, Hly$^-$	-
	BCS92	*efaAfs*$^+$	GelE$^-$, Hly$^-$	-
	P77	*efaAfs*$^+$, *gelE*$^+$	GelE$^+$, Hly$^-$	NIT, NOR, RIF, TEC, VAN
	GM22	*efaAfs*$^+$, *gelE*$^+$, *agg*$^+$	GelE$^+$, Hly$^-$	CIP, NOR
	GM26	*efaAfs*$^+$, *gelE*$^+$, *agg*$^+$	GelE$^+$, Hly$^-$	-
	GM33	*efaAfs*$^+$, *gelE*$^+$, *agg*$^+$	GelE$^+$, Hly$^-$	-
	CGM156	*efaAfs*$^+$	GelE$^-$, Hly$^-$	CIP, NIT, NOR, RIF, TEC, VAN
	CGM1514	*efaAfs*$^+$, *agg*$^+$	GelE$^-$, Hly$^-$	-
	CGM16	*efaAfs*$^+$, *gelE*$^+$, *agg*$^+$	GelE$^+$, Hly$^-$	CIP, NOR, RIF
	CGV16	*efaAfs*$^+$, *gelE*$^+$, *agg*$^+$	GelE$^+$, Hly$^-$	NOR
	SDP10	*efaAfs*$^+$, *gelE*$^+$, *agg*$^+$, *cylL*$_L$*L*$_S^+$, *cylL*$_L$*L*$_S$*M*$^+$	GelE$^+$, Hly$^+$	-
E. faecium	BNM58	n.d.	GelE$^-$, Hly$^-$	-
	SMA1	n.d.	GelE$^-$, Hly$^-$	CIP
	SMA7	n.d.	GelE$^-$, Hly$^-$	-
	SMA8	n.d.	GelE$^-$, Hly$^-$	-
	SMA101	n.d.	GelE$^-$, Hly$^-$	ERY, NIT
	SMA102	*efaAfs*$^+$	GelE$^-$, Hly$^-$	ERY, NIT
	SMA310	n.d.	GelE$^-$, Hly$^-$	ERY, NIT
	SMA320	*efaAfs*$^+$	GelE$^-$, Hly$^-$	ERY, NIT
	SMA361	*efaAfs*$^+$	GelE$^-$, Hly$^-$	ERY
	SMA362	n.d.	GelE$^-$, Hly$^-$	ERY, NIT
	SMA384	*gelE*$^+$	GelE$^-$, Hly$^-$	NIT
	SMA389	*gelE*$^+$	GelE$^-$, Hly$^-$	CIP, NIT, NOR
	SMF8	n.d.	GelE$^-$, Hly$^-$	-
	SMF39	*efaAfs*$^+$, *gelE*$^+$	GelE$^-$, Hly$^-$	-
	BCS59	n.d.	GelE$^-$, Hly$^-$	NIT
	BCS971	n.d.	GelE$^-$, Hly$^-$	ERY
	BCS972	n.d.	GelE$^-$, Hly$^-$	ERY
	B13	*gelE*$^+$	GelE$^+$, Hly$^-$	CIP
	B27	*efaAfs*$^+$, *gelE*$^+$	GelE$^+$, Hly$^-$	CIP
	MV5	*efaAfs*$^+$, *gelE*$^+$, *agg*$^+$	GelE$^-$, Hly$^-$	CIP, NIT
	P68	*efaAfs*$^+$, *gelE*$^+$, *cylL*$_L$*L*$_S^+$	GelE$^+$, Hly$^-$	CIP, NIT, NOR, RIF, TEC, VAN
	P623	*efaAfs*$^+$	GelE$^-$, Hly$^-$	ERY

Table 2 Preliminary safety evaluation of enterococci *(Continued)*

LPP29	n.d.	GelE$^-$, Hly$^-$	-
CV1	n.d.	GelE$^-$, Hly$^-$	-
CV2	n.d.	GelE$^-$, Hly$^-$	-
GM23	*efaAfs*$^+$	GelE$^-$, Hly$^-$	CIP, NOR, RIF, TET
GM29	*efaAfs*$^+$, *gelE*$^+$, *cylL*$_L$*L*$_S^+$	GelE$^-$, Hly$^-$	CIP, NOR, RIF
GM351	*efaAfs*$^+$, *gelE*$^+$, *agg*$^+$	GelE$^+$, Hly$^-$	CIP, NOR
GM352	*efaAfs*$^+$	GelE$^-$, Hly$^-$	CIP, NIT, NOR, RIF, TET
CGM171	n.d.	GelE$^-$, Hly$^-$	ERY
CGM172	*efaAfs*$^+$	GelE$^-$, Hly$^-$	ERY
TPM76	n.d.	GelE$^-$, Hly$^-$	-
TPP2	n.d.	GelE$^-$, Hly$^-$	-
NV50	*efaAfs*$^+$, *agg*$^+$	GelE$^-$, Hly$^-$	-
NV51	*efaAfs*$^+$	GelE$^-$, Hly$^-$	ERY
NV52	n.d.	GelE$^-$, Hly$^-$	ERY
NV54	*efaAfs*$^+$	GelE$^-$, Hly$^-$	ERY
NV56	*efaAfs*$^+$	GelE$^-$, Hly$^-$	-

[a]n.d., not detected.
[b]GelE and Hly refer to gelatinase and cytolysin/hemolysin activity, respectively.
[c]Abbreviation of antibiotics: CIP, ciprofloxacin; ERY, erythromycin; NIT, nitrofurantoin; NOR, norfloxacin; RIF, rifampicin; TEC, teicoplanin; TET, tetracycline; VAN, vancomycin.

38%), acquired antibiotic resistance (1 strain, 5%) or both (12 strains, 57%). Regarding *E. faecium* strains, 29 (76%) were dropped from further screening based on acquired antibiotic resistance (9 strains, 24%), the presence of virulence factors (3 strains, 8%) or both (17 strains, 45%).

Extracellular antimicrobial activity of the 49 pre-selected LAB

The antimicrobial activity of supernatants from the 49 pre-selected LAB (9 *E. faecium* selected based on their preliminary safety assessment and 40 non-enterococcal strains) with direct antimicrobial activity against fish pathogens was assayed against three indicator microorganisms by an ADT (Table 3). In this regard, 24 (49%) and 10 (20%) strains displayed extracellular antimicrobial activity in their supernatants and/or 20-fold concentrated supernatants against *Pediococcus damnosus* CECT4797 and *L. garvieae* JIP 29–99, respectively, but none of the strains inhibited the Gram-negative strain *A. hydrophila* CECT5734. Interestingly, the antimicrobial activity of the respective supernatants was sensitive to proteinase K treatment, but was not affected by the heat treatment, revealing the proteinaceous nature and heat stability of the secreted antimicrobial compounds (i.e., heat-stable bacteriocins). The 24 LAB strains secreting bacteriocins into the liquid growth medium belong to the species *P. pentosaceus* (15 strains), *E. faecium* (8 strains), and *Lb. curvatus* (1 strain).

In vitro safety assessment of the 49 pre-selected LAB

The 49 pre-selected LAB were further submitted to a comprehensive safety assessment by different *in vitro* tests.

Hemolysin production, bile salts deconjugation and mucin degradation

None of the non-enterococcal strains showed hemolytic activity, similarly as found for the 9 enterococci. Moreover, bile salts deconjugation and mucin degradation abilities were not found in any of the tested strains.

Enzymatic activities

The results of the analysis of enzymatic activity profiles of the tested LAB are shown in Table 4. None of the strains showed lipolytic activity, except *E. faecium* LPP29, TPM76, SMA7, and SMF8 which produced esterase (C4) and esterase lipase (C8). Moreover, none of the LAB strains showed protease activity (trypsin and α-chymotrypsin). Nevertheless, peptidase activity (leucine, valine or cystine arylamidase) was found in all the species. All strains showed acid phosphatase (except *E. faecium* TPM76 and *Lc. cremoris*) and naphthol-AS-BI-phosphohydrolase activities, but none displayed alkaline phosphatase activity. β-Galactosidase was found in most species (but not in all strains) except *Lb. curvatus* and *L. cremoris*. However, α-glucosidase was only found in the three *Lc. cremoris* strains. β-Glucosidase and N-acetyl-β-glucosaminidase activities were observed in most *E. faecium*, *Lactobacillus* spp., *L. cremoris*, and *P. pentosaceus* strains, but only in two *W. cibaria* strains, while the three *Lc. cremoris* strains showed β-glucosidase but lacked N-acetyl-β-glucosaminidase activity. On the other hand, α-galactosidase, β-glucuronidase, α-mannosidase, and

Table 3 Extracellular antimicrobial activity of the 49 pre-selected LAB[a]

LAB species[b]	Strain	Indicator microorganisms					
		P. damnosus CECT4797		*L. garvieae* JIP29-99		*A. hydrophila* CECT5734	
		S	CS	S	CS	S	CS
Enterococci							
E. faecium	BNM58	22.4	26.8	14.0	15.0	-	-
	SMA7	-	-	-	-	-	-
	SMA8	19.0	19.6	9.4	10.2	-	-
	SMF8	19.0	21.8	10.3	10.8	-	-
	LPP29	20.5	24.4	12.6	13.1	-	-
	CV1	15.0	19.2	-	-	-	-
	CV2	19.8	23.7	12.7	11.4	-	-
	TPM76	17.0	21.2	-	8.7	-	-
	TPP2	19.7	23.5	12.8	12.4	-	-
Non-enterococci							
Lb. curvatus	BCS35	18.2	24.7	-	-	-	-
P. pentosaceus	SMF120	-	-	-	-	-	-
	SMF130	7.4	9.7	-	-	-	-
	SMM73	-	9.5	-	-	-	-
	BCS46	-	9.4	-	-	-	-
	B5	8.1	9.0	-	-	-	-
	B11	-	9.0	-	-	-	-
	B41	7.3	11.7	-	-	-	-
	B260	7.3	10.6	-	-	-	-
	P63	-	9.8	-	-	-	-
	P621	-	10.5	-	-	-	-
	LPM78	-	8.3	-	-	-	-
	LPM83	7.9	11.0	-	-	-	-
	LPP32	8.5	11.3	-	8.9	-	-
	LPV46	8.2	11.3	-	8.2	-	-
	LPV57	7.6	10.5	-	-	-	-
	TPP3	9.0	11.7	7.5	9.2	-	-

[a]Antimicrobial activity (mm) of supernatants (S) and 20-fold concentrated supernatants (CS) as determined by an ADT.
[b]*Lb. carnosus*, *L. cremoris*, *Lc. cremoris* and *W. cibaria* strains did not show extracellular antimicrobial activity against any of the tested indicator microorganisms.

α-fucosidase activities were not detected in any of the tested LAB strains.

Antibiotic susceptibility determined by the broth microdilution test

The distribution of MICs of the tested antibiotics is summarized in Tables 5 and 6. Microbiological breakpoints for ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol reported by the FEEDAP document on the assessment of bacterial products used as feed additives in relation to antibiotic resistance [15] were used to categorise the 49 LAB as susceptible or resistant strains. In this document, the genus *Weissella*, which is considered a group of heterofermentative *Leuconostoc*-like LAB [16], is not included. For this reason, the respective MICs were interpreted by using the breakpoints given for the genus *Leuconostoc*. Besides, due to the lack of microbiological breakpoints for penicillin and linezolid on the FEEDAP document, we interpreted our results on these antibiotics according to the cut-off levels proposed by Klare *et al.* [17] for pediococci, namely 1 and 2 mg/L for penicillin and linezolid, respectively. According to our results, the percentages of strains showing antibiotic resistance in the genera *Weissella*, *Pediococcus*, *Lactobacillus* and *Enterococcus* were 60, 44, 33 and 11%, respectively, while none of the leuconostocs and lactococci showed this phenotype. In summary, 97.5% of the 40 non-enterococcal

Table 4 Enzymatic activity profiles of the 49 pre-selected LAB[a]

Species	Strain	Esterase (C4)	Esterase lipase (C8)	Leucine arylamidase	Valine arylamidase	Cystine arylamidase	Acid phosphatase	Naphthol-AS-BI-phosphohydrolase	β-Galactosidase	α-Glucosidase	β-Glucosidase	N-acetyl-β-glucosaminidase
Enterococci												
E. faecium	BNM58	0	0	≥40	10	10	20	10	0	0	0	0
	SMA7	20	20	≥40	30	20	30	10	0	0	0	0
	SMA8	0	0	≥40	≥40	5	5	5	5	0	20	≥40
	SMF8	5	5	10	5	5	20	10	0	0	30	0
	LPP29	10	10	30	5	20	10	10	0	0	0	0
	CV1	0	0	≥40	≥40	5	10	20	20	0	30	≥40
	CV2	0	0	≥40	≥40	10	10	20	0	0	10	≥40
	TPM76	30	10	20	0	0	0	10	10	0	0	0
	TPP2	0	0	≥40	20	10	10	10	5	0	30	0
Non-enterococci												
Lb. carnosus	SMA17	0	0	≥40	≥40	0	30	20	30	0	30	30
	B43	0	0	≥40	≥40	0	5	5	10	0	0	0
Lb. curvatus	BCS35	0	0	≥40	10	5	10	20	0	0	5	10
L. cremoris	SMF110	0	0	≥40	≥40	0	20	20	0	0	30	30
	SMF161	0	0	20	0	5	≥40	20	0	0	0	0
	SMF166	0	0	≥40	≥40	0	20	20	0	0	10	10
Lc. cremoris	SMM69	0	0	10	0	0	0	10	≥40	30	≥40	0
	BCS251	0	0	5	0	0	0	5	20	20	10	0
	BCS252	0	0	10	0	0	0	10	30	20	10	0
P. pentosaceus	SMF120	0	0	≥40	≥40	20	≥40	≥40	0	0	20	20
	SMF130	0	0	≥40	≥40	20	30	≥40	20	0	≥40	≥40
	SMM73	0	0	≥40	30	10	20	30	20	0	30	≥40
	BCS46	0	0	≥40	≥40	5	20	30	30	0	≥40	≥40
	B5	0	0	30	≥40	10	10	20	10	0	30	≥40
	B11	0	0	≥40	30	0	5	20	0	0	30	≥40
	B41	0	0	30	≥40	0	5	20	5	0	20	≥40
	B260	0	0	≥40	≥40	10	20	30	0	0	20	30
	P63	0	0	≥40	≥40	5	20	20	30	0	30	≥40
	P621	0	0	≥40	≥40	0	5	30	0	0	30	≥40
	LPM78	0	0	30	30	5	10	20	20	0	30	≥40

Table 4 Enzymatic activity profiles of the 49 pre-selected LAB[a] *(Continued)*

	LPM83	0	0	30	30	5	10	20	30	0	10	≥40
	LPP32	0	0	≥40	≥40	5	5	20	0	0	30	≥40
	LPV46	0	0	≥40	≥40	5	20	30	5	0	30	30
	LPV57	0	0	≥40	≥40	5	20	30	30	0	≥40	≥40
	TPP3	0	0	≥40	≥40	5	5	5	10	0	0	0
W. cibaria	BNM69	0	0	0	0	0	30	10	30	0	0	0
	SMA14	0	0	0	0	0	20	5	10	0	0	0
	SMA25	0	0	≥40	≥40	0	30	20	≥40	0	30	30
	BCS50	0	0	0	0	0	30	20	30	0	0	0
	B4620	0	0	20	20	0	30	20	30	0	5	5
	P38	0	0	0	0	0	≥40	20	≥40	0	0	0
	P50	0	0	0	0	0	≥40	20	0	0	0	0
	P61	0	0	0	0	0	20	10	0	0	0	0
	P64	0	0	0	0	0	30	10	0	0	0	0
	P69	0	0	0	0	0	≥40	20	≥40	0	0	0
	P71	0	0	0	0	0	≥40	10	0	0	0	0
	P73	0	0	0	0	0	30	20	30	0	0	0
	P622	0	0	0	0	0	≥40	10	0	0	0	0
	SDM381	0	0	10	5	0	20	10	30	0	0	0
	SDM389	0	0	0	0	0	≥40	20	≥40	0	0	0

[a]Enzymatic activities determined by an APIZYM test. Relative activity between 0 and ≥ 40 nmol.

strains resulted susceptible to ampicillin, 100% to gentamicin, 72.5% to kanamycin, 100% to streptomycin, 95% to erythromycin, 87.5% to clindamycin, 95% to tetracycline, and 100% to chloramphenicol. For vancomycin, it is known that facultative and obligate heterofermentative *Lactobacillus, Pediococcus* spp. and *Leuconostoc* spp. are intrinsically resistant. In contrast, the three lactococci were clearly susceptible to these antibiotics, showing a MIC of 0.5 mg/L. On the other hand, according to the cut-off values proposed by Klare *et al.* [17], 93% of *P. pentosaceus* strains were susceptible to penicillin and linezolid. With regard to *E. faecium*, all the tested strains were susceptible to ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, tetracycline, chloramphenicol, and erythromycin except *E. faecium* BNM58 against the latter antibiotic (MIC = 8 mg/L). Moreover, multiple antibiotic resistance (three antibiotics) was only detected in *P. pentosaceus* LPM78 (6.2%) and *W. cibaria* SMA25 (6.7%).

Detection of antibiotic resistance genes

The non-enterococcal strains showing antibiotic resistances in the VetMIC assays (17 strains) were further submitted to PCR in order to identify the presence of the respective antibiotic resistance genes. The tested strains were the following: *Lb. carnosus* B43 (ampicillin resistant), *P. pentosaceus* TPP3 and SMF120 (tetracycline resistant), *P. pentosaceus* LPP32, LPM83 and B5 (clindamycin resistant), *P. pentosaceus* LPV57 and *W. cibaria* P50, P61, P64, P73, SDM381, SDM389, SMA14 and BCS50 (kanamycin resistant), and *P. pentosaceus* LPM78 and *W. cibaria* SMA25 (kanamycin, erythromycin and clindamycin resistant). Acquired antibiotic resistances likely due to added genes were only found in strains within the genera *Pediococcus* (12.5%) and *Weissella* (6.7%). The genes involved in the horizontal transfer of resistance to tetracycline [*tet*(K), *tet*(L) and *tet*(M)], kanamycin [*aac(6´)-Ie-aph(2´´)-Ia*] and erythromycin [*erm*(A), *erm*(B) and *erm*(C)] were not detected. However, *P. pentosaceus* LPM78 and *W. cibaria* SMA25 harboured the erythromycin resistance gene *mef*(A/E). The obtained amplicons were sequenced and found to have 99% homology with the macrolide-efflux protein (*mef*E) gene described for *Streptococcus pneumoniae* and other *Streptococcus* spp. Moreover, *P. pentosaceus* LPM78 and LPM83 harboured the *lnu*(A) gene encoding the lincosamide *O*-nucleotidyltransferase that inactivates lincomycin and clindamycin. Sequencing of both amplicons showed 97% and 93% homology with lincosamide nucleotidyltransferase [*lnu*(A)] gene described for *Staphylococcus haemolyticus* and *S. aureus*, respectively. Nevertheless, *lnu*(B) was not detected in any of the tested strains. With regard to *E. faecium* BNM58, which was phenotypically resistant to erythromycin, none of the respective genes [*erm*(A), *erm*(B), *erm*(C) and *mef*(A/E)] were detected.

Discussion

In this work, the antimicrobial activity against fish pathogens and the *in vitro* safety of 99 LAB previously isolated from fish, seafood and fish products [14] have been assayed by using microbiological, biochemical and genetic assays in order to identify and select the most suitable candidates to be further evaluated as probiotics for a sustainable aquaculture. LAB are widely known for their ability to inhibit bacterial pathogens by the production of antimicrobial compounds such as organic acids, oxygen peroxide and ribosomally-synthesized peptides referred to as bacteriocins, which constitutes a desirable property for probiotics and a sustainable alternative to antibiotics [9,18]. In this respect, most of the LAB of aquatic origin tested in this work displayed a broad antimicrobial spectrum against the main Gram-positive and

Table 5 MICs distribution of 10 antibiotics for the 9 enterococcal strains

Antibiotics	Number of strains with the indicated MIC (mg/L)[a]																EFSA breakpoints (mg/L)[b]
	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	
Ampicillin			5	3	1												**2**
Vancomycin				9													**4**
Gentamicin					4		5										**32**
Kanamycin								1		2	4	2					**1024**
Streptomycin							1		3	5							**128**
Erythromycin			5				3	**1**									**4**
Tetracycline					9												**4**
Chloramphenicol							8	1									**16**
Linezolid						9											**n.a.**
Narasin			1	8													**n.a.**

[a]MICs determined by a VetMIC test. The antibiotic dilution ranges were: 0.25-32 mg/L (ampicillin), 1-128 mg/L (vancomycin), 2-256 mg/L (gentamicin), 16-2048 mg/L (kanamycin), 8-1024 mg/L (streptomycin), 0.5-64 mg/L (erythromycin, tetracycline and chloramphenicol), 0.25-16 mg/L (linezolid) and 0.12-16 (narasin). MICs which exceeded the upper or lower limit of the tested range are listed in the next dilution series. MICs higher than the EFSA breakpoints are indicated in bold.
[b]LAB with MICs higher than the EFSA breakpoints are considered as resistant strains [15]. n.a., not available.

Table 6 MICs distribution of 15 antibiotics for the 40 non-enterococcal strains

Antibiotics	Species (no. of tested isolates)	Number of strains with the indicated MIC (mg/L)[a]																	EFSA breakpoints (mg/L)[b]
		0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	
Ampicillin	*Lb. carnosus* (2)									1	**1**									**4**
	Lb. curvatus (1)						1													**4**
	L. cremoris (3)				1	2														**2**
	Lc. cremoris (3)				1	2														**2**
	P. pentosaceus (16)								15	1										**4**
	W. cibaria (15)						15													**n.a.**
Vancomycin	*Lb. carnosus* (2)										2									**n.r.**
	Lb. curvatus (1)											1								**n.r.**
	L. cremoris (3)						3													**4**
	Lc. cremoris (3)															3				**n.r.**
	P. pentosaceus (16)															16				**n.r.**
	W. cibaria (15)															15				**n.a.**
Gentamicin	*Lb. carnosus* (2)						1		1											**16**
	Lb. curvatus (1)									1										**16**
	L. cremoris (3)					3														**32**
	Lc. cremoris (3)					3														**16**
	P. pentosaceus (16)					1		1	9	3	2									**16**
	W. cibaria (15)					6		7	1		1									**n.a.**
Kanamycin	*Lb. carnosus* (2)								1		1									**64**
	Lb. curvatus (1)											1								**64**
	L. cremoris (3)								2	1										**64**
	Lc. cremoris (3)										1	2								**16**
	P. pentosaceus (16)										1			13	**2**					**64**
	W. cibaria (15)									1	1	4	4	4	1					**n.a.**
Streptomycin	*Lb. carnosus* (2)										1		1							**64**
	Lb. curvatus (1)												1							**64**
	L. cremoris (3)										2	1								**32**
	Lc. cremoris (3)										1	2								**64**
	P. pentosaceus (16)											1	5	10						**64**
	W. cibaria (15)									2		7	5	1						**n.a.**
Erythromycin	*Lb. carnosus* (2)				2															**1**
	Lb. curvatus (1)				1															**1**
	L. cremoris (3)			2	1															**1**
	Lc. cremoris (3)			1	2															**1**
	P. pentosaceus (16)			1	4	7		3				**1**								**1**
	W. cibaria (15)					9	5				1									**n.a.**
Clindamycin	*Lb. carnosus* (2)		1		1															**1**
	Lb. curvatus (1)	1																		**1**
	L. cremoris (3)	2			1															**1**

Table 6 MICs distribution of 15 antibiotics for the 40 non-enterococcal strains *(Continued)*

	Lc. cremoris (3)	2		1											**1**
	P. pentosaceus (16)	3	2	7					**1**	**3**					**1**
	W. cibaria (15)	2			6	5	1		1						**n.a.**
Tetracycline	*Lb. carnosus* (2)							1	1						**8**
	Lb. curvatus (1)							1							**8**
	L. cremoris (3)					1	1	1							**4**
	Lc. cremoris (3)							1	2						**8**
	P. pentosaceus (16)								1		13	**2**			**8**
	W. cibaria (15)									15					**n.a.**
Chloramphenicol	*Lb. carnosus* (2)							1	1						**4**
	Lb. curvatus (1)								1						**4**
	L. cremoris (3)								1	2					**8**
	Lc. cremoris (3)								3						**4**
	P. pentosaceus (16)							1	5	10					**4**
	W. cibaria (15)									15					**n.a.**
Neomycin	*Lb. carnosus* (2)							1		1					**n.a.**
	Lb. curvatus (1)									1					**n.a.**
	L. cremoris (3)					2		1							**n.a.**
	Lc. cremoris (3)					3									**n.a.**
	P. pentosaceus (16)					1			9	4	2				**n.a.**
	W. cibaria (15)					4		6	4		1				**n.a.**
Penicillin	*Lb. carnosus* (2)								1	1					**n.a.**
	Lb. curvatus (1)					1									**n.a.**
	L. cremoris (3)					3									**n.a.**
	Lc. cremoris (3)				1	2									**n.a.**
	P. pentosaceus (16)						7	8	1						**n.a.**
	W. cibaria (15)							7	7		1				**n.a.**
Linezolid	*Lb. carnosus* (2)							2							**n.a.**
	Lb. curvatus (1)								1						**n.a.**
	L. cremoris (3)							1	2						**n.a.**
	Lc. cremoris (3)						1	2							**n.a.**
	P. pentosaceus (16)								15	1					**n.a.**
	W. cibaria (15)								15						**n.a.**
Ciprofloxacin	*Lb. carnosus* (2)								2						**n.a.**
	Lb. curvatus (1)										1				**n.a.**
	L. cremoris (3)								2	1					**n.a.**
	Lc. cremoris (3)								1	2					**n.a.**
	P. pentosaceus (16)												16		**n.a.**
	W. cibaria (15)									5	10				**n.a.**
Rifampicin	*Lb. carnosus* (2)					1	1								**n.a.**

Table 6 MICs distribution of 15 antibiotics for the 40 non-enterococcal strains *(Continued)*

	Lb. curvatus (1)	1													**n.a.**
	L. cremoris (3)								1	2					**n.a.**
	Lc. cremoris (3)			1	2										**n.a.**
	P. pentosaceus (16)				2	13	1								**n.a.**
	W. cibaria (15)							12	3						**n.a.**
Trimethoprim	*Lb. carnosus* (2)									1		1			**n.a.**
	Lb. curvatus (1)							1							**n.a.**
	L. cremoris (3)											3			**n.a.**
	Lc. cremoris (3)					1	2								**n.a.**
	P. pentosaceus (16)							8	8						**n.a.**
	W. cibaria (15)											15			**n.a.**

[a]MICs determined by a VetMIC test. The antibiotic dilution ranges were: 0.03-16 mg/L (ampicillin, clindamycin, penicillin and linezolid), 0.25-128 mg/L (vancomycin and ciprofloxacin), 0.5-256 mg/L (gentamicin, streptomycin and neomycin), 2-1024 mg/L (kanamycin), 0.016-8 mg/L (erythromycin), 0.12-64 (tetracycline, chloramphenicol, rifampicin and trimethoprim). MICs which exceeded the upper or lower limit of the tested range are listed in the next dilution series. MICs higher than the EFSA breakpoints are indicated in bold.
[b]LAB with MICs higher than the EFSA breakpoints are considered as resistant strains [15]. n.r., not required; n.a., not available.

Gram-negative fish pathogens, being remarkable that a high number of strains (24 out of 49 strains, 49%) were identified as potential bacteriocin producers. Recently, bacteriocin production ability has been proposed as a key property for selection of probiotic LAB to be used in aquaculture as an alternative to antibiotics to fight against fish pathogen infections [19], similarly as proposed for human and farm animal probiotics [20-22]. In aquaculture farming, lactococcosis produced by the zoonotic agent *L. garvieae*, causing hemorrhagic septicaemia and meningoencephalitis, is one of the most serious diseases affecting several marine and fresh water fish species [23]. With regard to this, our work shows that putative bacteriocinogenic LAB active against this relevant fish pathogen are common amongst the microbiota isolated from aquatic animals (10 strains, 20%).

The application of probiotics in aquaculture may modify the microbial ecology of the aquatic hosts and their surrounding environment, and thus the assessment of their safety to the target aquatic species, the environment and humans constitutes an essential issue [24]. To date, several studies describing the screening and evaluation of LAB as probiotic candidates for aquaculture have been reported [25-28]; however, the safety assessment of the strains is generally limited to *in vivo* challenge tests and rearing trials in order to confirm their lack of toxicity to the aquatic hosts [24,25,28-31]. Strikingly, *in vitro* safety assessment studies have not been generally addressed, despite they have lower economic and ethic costs and result very effective to evaluate the safety of a high number of candidate probiotic strains not only for the host species, but also for humans and the environment. According to EFSA [13], most of the LAB species tested in this work (*P. pentosaceus*, *Lb. curvatus*, *L. lactis*, *Lc. mesenteroides*) are included in the QPS list and, therefore, demonstration of their safety only requires confirmation of the absence of determinants of resistance to antibiotics of human and veterinary clinical significance. However, in the case of enterococci, a more thorough, strain-specific evaluation is required to assess the risk associated to their intentional use in the food chain, while no guidelines are given for the safety assessment of the species *W. cibaria* [13].

Our results show that enterococcal virulence factors were more frequently found in *E. faecalis* than in *E. faecium*, which is in concordance with previous reports [32-34]. In this respect, most of the *E. faecalis* (95%) and a large percentage of the *E. faecium* (53%) strains evaluated in this work showed, at least, one virulence factor, being *efaAfs*, *gelE* and *agg* the most frequently detected genes. With regard to *gelE*, which encodes for an extracellular zinc endopeptidase that hydrolyzes gelatin, collagen, hemoglobin, and other bioactive compounds, this gene was detected at high frequency in *E. faecalis*, with all the $gelE^+$ strains showing gelatinase activity. However, five out of nine *E. faecium* strains harbouring *gelE* were unable to degrade gelatin, suggesting the carriage of a non-functional gene, as previously reported [32,33]. Likewise, in the case of *E. faecium* P68 and *E. faecium* GM29 harbouring $cylL_L$-$cylL_S$, the lack of hemolytic activity may be explained by the absence of *cylM*, whose product is involved in the post-translational modification of cytolysin. On the other hand, *esp* and *hyl*, which encode a cell wall-associated protein involved in immune evasion and an hyaluronidase enzyme, respectively, were not found in any of the tested LAB. Previous studies have reported that *esp* and *hyl* are more common in ampicillin-resistant/vancomycin-resistant *E. faecium* (VREF) than in ampicillin-susceptible/VREF

strains [35]. In this context, the increase in the incidence of VREF at hospital settings has been attributed mainly to the spread of ampicillin-resistant VREF exhibiting *esp* and/or *hyl* [36,37]. Therefore, the fact that the *E. faecium* strains evaluated in this work lack these genes might be related with their non-clinical origin and absence of ampicillin resistance.

The use and frequent overuse of antibiotics, including those used in human medicine, in fish farming has resulted in the emergence and spread of antibiotic-resistant bacteria in the aquaculture environment. This possesses a threat to human and animal health due to the increase of acquired antibiotic resistance in fish pathogens, the transfer of their genetic determinants to bacteria of terrestrial animals and to human pathogens, and the alterations of the bacterial microbiota of the aquatic environment [11,29]. In our study, the percentage of enterococcal strains showing acquired antibiotic resistance was 68%. Interestingly, the results found in *E. faecium* (71%) and *E. faecalis* (62%) were similar, however, higher percentages of resistance to ciprofloxacin and/or norfloxacin, rifampicin, and glycopeptides were observed in *E. faecalis*. Nevertheless, the occurrence of erythromycin and tetracycline resistance was frequently detected amongst *E. faecium* (45%) but only in one *E. faecalis* strain (5%). In spite of the high prevalence of acquired antibiotic resistance found in enterococci of aquatic origin, they showed low incidence or absence of resistance to the clinically relevant antibiotics vancomycin (8.5%) and ampicillin, penicillin and gentamicin, respectively, which is in agreement with previous studies [33,38]. Moreover, the percentages of strains showing antibiotic resistance in the genera *Weissella*, *Pediococcus* and *Lactobacillus* were 60, 44 and 33%, respectively, while none of the leuconostocs and lactococci showed this phenotype. In this regard, our results indicate that the LAB susceptibility patterns of MIC values to clinically relevant antibiotics are species-dependent, similarly as previously described by other authors [39,40]. Moreover, multiple antibiotic resistance was commonly found in strains within the genus *Enterococcus* (37%), mainly in *E. faecalis*, while being very infrequent in the non-enterococcal strains (5%).

According to EFSA [29], the determination of MICs above the established breakpoint levels, for one or more antibiotic, requires further investigation to make the distinction between added genes (genes acquired by the bacteria via gain of exogenous DNA) or to the mutation of indigenous genes. According to our results, acquired antibiotic resistance likely due to added genes is not a common feature amongst the non-enterococcal LAB of aquatic origin (7.5%). In this respect, this genotype was only found in the genera *Pediococcus* (12.5%) and *Weissella* (6.7%). Although *P. pentosaceus* LPV57 and LPM78 showed resistance to kanamycin (MIC of 128 mg/L), the respective resistance gene [*aac(6´)-Ie-aph(2´´)-Ia*] was not found in these strains. Similarly, *P. pentosaceus* TPP3 and SMF120 were phenotypically resistant to tetracycline (MIC of 16 mg/L), but did not contain *tet (K), tet(L)* or *tet(M)*. In this respect, Ammor *et al.* [41] reported that pediococci are intrinsically resistant to the latter two antibiotics, as well as to glycopeptides (vancomycin and teicoplanin), streptomycin, ciprofloxacin and trimethoprim-sulphamethoxazole. Other authors proposed a MIC for tetracycline in pediococci ranging between 8 and 16 mg/L [42], or of 32 mg/L for oxytetracycline in *P. pentosaceus* [17]. The tetracycline breakpoints suggested for pediococci by EFSA are lower than the MICs observed in our work and others [17,42]. On the other hand, the only antibiotic resistance detected in *Leuconostoc* strains was for vancomycin, which is an intrinsic property of this genus. It has been previously reported that *Leuconostoc* strains display poor, if any, resistance to antibiotics of clinical interest [38]. With regard to lactococci, the three *L. cremoris* strains evaluated were susceptible to all the antibiotics; however, relatively high MICs for rifampicin (16–32 mg/L) and trimethoprim (≥ 64 mg/L) were detected. In fact, most lactococcal species are resistant to trimethoprim [41]. As expected, all strains of heterofermentative *Lactobacillus* spp. were resistant to vancomycin but susceptible to the rest of the assayed antibiotics, except *Lb. carnosus* B43, which showed the highest MIC for ampicillin and penicillin (MICs of 8 and 4 mg/L, respectively). In this context, the presence of modifications in the low affinity penicillin-binding protein (PBP) that confers resistance to penicillin and β-lactams in *E. faecium* and *Streptococcus pneumoniae*, has been reported [43,44]. Moreover, nine PBPs have been described in *Lb. casei* ATCC 393 [45], which leads us to suggest that a similar mechanism may be also responsible for the ampicillin and penicillin resistance found in *Lb. carnosus* B43. The resistance to vancomycin detected in *Pediococcus*, *Leuconostoc* and *Lactobacillus* species in this study might be due to the presence of D-Ala-D-Lactate in their peptidoglycan rather than D-Ala-D-Ala dipeptide [46]. In this context, all tested *W. cibaria* strains showed MICs ≥ 128 mg/L for vancomycin, suggesting that vancomycin resistance is an intrinsic property of this species. In relation to *Weissella* spp., studies on antibiotic resistance profiles are very limited [47] and breakpoints have not been defined by EFSA [15]. In our study, most *W. cibaria* strains showed low MIC values; however *W. cibaria* BCS50 showed relatively high MICs for penicillin (8 mg/L) and kanamycin (64 mg/L), and *W. cibaria* SMA25 showed MICs of 128 mg/L for kanamycin, 8 mg/L for gentamicin, erythromycin and neomycin, and 2 mg/L for clindamycin. Therefore, these two strains were discarded of this study, while *W. cibaria* P50, P61, P64, P73, SMA14, SDM381 and SDM389 were not included in the final selection due to their MICs for kanamycin (32–

64 mg/L). According to these results, as a rule of thumb, we propose for *W. cibaria* the breakpoints assigned to *Leuconostoc* spp. by EFSA [15], until further studies establish the wild-type MIC ranges within this species. In spite of that, different MICs for rifampicin and trimethoprim for *W. cibaria* and *Lc. cremoris* were found in this study. The reduced susceptibility of *W. cibaria* towards trimethoprim could indicate an intrinsic resistance to this antibiotic [48]. In our work, the only antibiotic resistance genes found were *mef*(A/E), which encodes a drug efflux pump conferring a low to moderate level of resistance to 14 (erythromycin and clarithromycin)- and 15 (azithromycin)-membered macrolides but not to lincosamide or streptogramin B antibiotics [49], and *lnu*(A), encoding the lincosamide *O*-nucleotidyltransferase that inactivates lincomycin and clindamycin [50]. In this respect, *P. pentosaceus* LPM78 and *W. cibaria* SMA25, displaying erythromycin resistance (MIC = 8 and ≥ 8 mg/L, respectively), carried the gene *mef*(A/E), which can be found in a variety of Gram-positive bacteria, including corynebacteria, enterococci, micrococci, and several streptococcal species [51,52]. On the other hand, two pediococci (*P. pentosaceus* LPM78 and LPM83) that showed resistance to clindamycin (MIC = 4 and 2 mg/L, respectively) carried the gene *lnu*(A), which had been only previously found in staphylococci, streptococci, enterococci and lactobacilli of animal origin and in staphylococci isolated from humans [50,53]. Strikingly, the clindamycin resistant strains *P. pentosaceus* LPP32 and B5 and *W. cibaria* SMA25 (MIC = 4 and 2 mg/L, respectively) did not harbour this gene nor *lnu*(B). To our knowledge, this is the first description of *mef*(A/E) in the genera *Pediococcus* and *Weissella*, and *lnu*(A) in the genus *Pediococcus*. The detection of resistance genes for macrolide and lincosamide in non-enterococcal strains suggests a wider distribution of this group of genes than previously anticipated.

The *in vitro* subtractive screening proposed in this work also include the assessment of bile salts deconjugation, mucin degradation, biogenic amine production and other potentially detrimental enzymatic activities such as the β-glucuronidase activity, which should be absent in probiotic candidates [54-56]. Excessive deconjugation of bile salts may be unfavourable in animal production since unconjugated bile acids are less efficient than their conjugated counterparts in the emulsification of dietary lipids. In addition, the formation of micelles, lipid digestion and absorption of fatty acids and monoglycerides could be impaired by deconjugated bile salts [57]. Similarly, excessive degradation of mucin may be harmful as it may facilitate the translocation of bacteria to extraintestinal tissues [55]. In this respect, it is worthy to note that none of the 49 tested LAB deconjugated bile salts nor exhibited mucinolytic activity, the latter indicating their low invasive and toxigenic potential at the mucosal barrier. These results are in accordance with previous findings showing that LAB do not degrade mucin *in vitro* [58,59]. Moreover, β-glucuronidase activity has been associated with the generation of potential carcinogenic metabolites [56]; however, none of the LAB tested in our study displayed this harmful enzymatic activity. In a previous work [60], we demonstrated that none of the 40 non-enterococcal strains evaluated herein produced histamine, tyramine or putrescine. With regard to enterococci, the nine *E. faecium* strains only produced tyramine, being *E. faecium* CV1 a low producer of this biogenic amine. Although the lack of biogenic amine production by probiotic strains is a desirable trait, it should be borne in mind that tyramine production by enterococci is a very frequent trait [60,61]. Finally, several studies have suggested that probiotic microorganisms might exert a beneficial effect in the digestion process of fish due to the production of extracellular enzymes [62-65]. In our work, the LAB strains of aquatic origin within the genera *Pediococcus*, *Enterococcus* and *Lactobacillus* showed a higher number of enzymatic activities than *Lactococcus*, *Leuconostoc* and *Weissella*, being the enzymatic profiles similar amongst strains within the same genus. In this respect, nearly all the strains produced phosphatases, which might be involved in nutrient absorption [64], and peptidases and glucosidases that breakdown peptides and carbohydrates, respectively. However, the tested LAB showed weak lipolytic activity and no proteolytic activity.

Conclusions

This work shows that antimicrobial/bacteriocin activity against fish pathogens is a widespread probiotic property amongst LAB isolated from aquatic animals regarded as human food. However, particular safety concerns based on antibiotic resistances and virulence factors were dominant within *E. faecalis* (100%) and *E. faecium* (79%), and acquired antibiotic resistance genes were not commonly found (7.5%; erythromycin and clindamycin) amongst the non-enterococcal isolates of aquatic origin. To our knowledge, this is the first large-scale study describing the antimicrobial activity against fish pathogens and the safety assessment beyond the QPS approach of LAB isolated from aquatic animals. The *in vitro* subtractive screening presented herein, which allowed the selection of 33 strains (8 *E. faecium*, 11 *P. pentosaceus*, 1 *Lb. carnosus*, 1 *Lb. curvatus*, 3 *L. cremoris*, 3 *Lc. cremoris* and 6 *W. cibaria*) out of 99 LAB isolates of aquatic origin, constitutes a valuable strategy for the large-scale preliminary selection of putatively safe LAB intended for use as probiotics in aquaculture and to avoid the spreading of bacterial cultures with harmful traits into the aquatic environment. Nevertheless, a comprehensive *in vivo* assessment of their lack of toxicity and undesirable effects must be also carried out using cell lines, live food and, ultimately, aquatic animals

before their unequivocal consideration as safe probiotics for a sustainable aquaculture.

Methods

Bacterial strains and growth conditions

A total of 99 LAB (59 enterococci and 40 non-enterococci) of aquatic origin with antimicrobial activity against spoilage and food-borne pathogenic bacteria of concern for the fish industry, previously isolated and identified by our group from fish, seafood and fish products [14], were used in this study (Table 1). The LAB strains were isolated on non-supplemented MRS (Oxoid, Ltd., Basingstoke, United Kingdom) or KAA (Oxoid) agar (1,5%, w/v) at 25°C, and taxonomically identified [14] by sequencing of the genes encoding 16S rRNA (*16S rDNA*) [66] and/or superoxide dismutase (*sodA*) [67]. Unless otherwise stated, LAB were grown aerobically in MRS broth at 32°C.

Direct antimicrobial activity assay

The antimicrobial activity of the 99 LAB against the main Gram-positive and Gram-negative fish pathogens was assayed by a qualitative stab-on-agar test (SOAT) as previously described by Cintas *et al*. [68]. Briefly, pure cultures were stabbed onto MRS or Tryptone Soya Agar (TSA) (Oxoid) plates supplemented with glucose (2%, w/v) and incubated at 32°C for 5 h, and then 40 ml of the corresponding soft agar (0.8%, w/v) medium containing about 1×10^5 CFU/ml of the indicator strain was poured over the plates. After incubation at 28-37°C for 16–24 h depending on the indicator strain, the plates were checked for inhibition zones (absence of visible microbial growth around the stabbed cultures), and only inhibition halos with diameters >3 mm were considered positive. *L. garvieae* JIP29-99 was grown aerobically in Tryptone Soya Broth (TSB) (Oxoid) at 37°C. *S. agalactiae* CF01173 and *S. iniae* LMG14521 were grown aerobically in Brain Heart Infusion (BHI) broth (Oxoid) at 37°C. *A. hydrophila* CECT5734, *Ls. anguillarum* CECT4344, *Ls. anguillarum* CECT7199, and *Ph. damselae* CECT626 strains were grown aerobically in TSB at 28°C. *V. alginolyticus* CECT521 was grown aerobically in TSB supplemented with NaCl (1%, w/v; Panreac Química S.A.U, Barcelona, Spain) at 28°C.

Extracellular antimicrobial activity assay

The antimicrobial activity of supernatants from LAB cultures grown in MRS broth at 32°C for 16 h was determined by an agar well-diffusion test (ADT) as previously described by Cintas *et al.* [68]. Supernatants were obtained by centrifugation of cultures at $10{,}000 \times g$ at 4°C for 10 min, adjusted to pH 6.2 with 1 M NaOH, filter-sterilized through 0.22 μm-pore-size filters (Millipore Corp., Bedford, Massachussets, USA) and stored at −20°C until use. Fifty-μl aliquots of cell-free culture supernatants were placed into wells (6-mm diameter) cut in cooled MRS or TSB agar (0.8%, wt/vol) plates previously seeded (1×10^5 CFU/ml) with the indicator microorganisms *Pediococcus damnosus* CECT4797, *L. garvieae* JIP29-99 or *A. hydrophila* CECT5734. After 2 h at 4°C, the plates were incubated under the same conditions mentioned above to allow for the growth of the target microorganisms and then analyzed for the presence of inhibition zones around the wells. To determine the proteinaceous nature of the antimicrobial compounds, supernatants showing antimicrobial activity were subjected to proteinase K treatment (10 mg/ml) (AppliChem GmbH, Germany) at 37°C for 2 h. After proteinase K inactivation by heat treatment (100°C, 10 min), samples were assayed for residual antimicrobial activity by an ADT as described above using *P. damnosus* CECT4797 as indicator microorganism. Supernatants with no added enzyme were treated as indicated above and used as controls. For further characterization of the antimicrobial compounds, 7 ml of supernatants from an overnight culture of LAB were subjected to peptide concentration by ammonium sulphate precipitation. Ammonium sulphate was gradually added to the supernatants to achieve 50% saturation. Samples were kept at 4°C with stirring for 3 h, and then centrifuged at $10{,}000 \times g$ at 4°C for 30 min. Pellets and floating solid material were combined and solubilized in 350 μl of 20 mM sodium phosphate (pH 6.0), and antimicrobial activity of the resulting 20-fold concentrated supernatants was determined by an ADT as described above.

PCR detection of potential virulence factors in enterococci

Detection of genes encoding potential virulence factors in the 59 enterococci was performed by PCR. The following primer pairs were used: TE3/TE4 for detection of *agg* (aggregation substance), TE9/TE10 for *gelE* (gelatinase), TE34/TE36 for *esp* (enterococcal surface protein), TE5/TE6 for *efaAfs* (*Enterococus faecalis* endocarditis antigen) [32], HYLn1/HYLn2 for *hyl* (hyaluronidase) [35], $CYLL_L$–R1/$CYLL_S$–R2 for $cylL_L$–$cylL_S$ (cytolysin precursor) [69], and RHCT1/RHCT2 for $cylL_L$–$cylL_S$-*cylM* (cytolysin precursor and posttranslational modifier) [70]. Oligonucleotide primers were obtained from Sigma-Genosys Ltd. (Cambridge, United Kingdom). The positive control strains for detection of potential virulence factors were the following: *E. faecalis* P4 for $cylL_L$–$cylL_s$, $cylL_L$–$cylL_S$–*cylM*, *agg*, *gelE* and *efaAfs*, *E. faecalis* P36 for *esp* [32], and *E. faecium* C68 for *hyl* [35]. PCR-amplifications were performed from total bacterial DNA obtained using the Wizard DNA Purification Kit (Promega, Madrid, Spain) in 25 μl reaction mixtures with 1 μl of purified DNA, 0.7 μM of each primer, 0.2 mM of each dNTP, buffer 1×, 1.5 mM $MgCl_2$ and 0.75 U of Platinum Taq DNA polymerase (Invitrogen, Madrid, Spain). Samples were subjected to an initial cycle of denaturation (97°C for 2 min), followed by 35 cycles of denaturation (94°C for

45 s), annealing (48 to 64°C for 30 s) and elongation (72°C for 30 to 180 s), ending with a final extension step at 72°C for 7 min in an Eppendorf Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). PCR products were analyzed by electrophoresis on 1-2% (w/v) agarose (Pronadisa, Madrid, Spain) gels stained with Gel red (Biotium, California, USA), and visualized with the Gel Doc 1000 documentation system (Bio-Rad, Madrid, Spain). The molecular size markers used were HyperLadder II (Bioline GmbH, Germany) and 1Kb Plus DNA ladder (Invitrogen).

Production of gelatinase by enterococci

Gelatinase production was determined using the method previously described by Eaton and Gasson [32]. Briefly, enterococci were grown in MRS broth overnight at 32°C, and streaked onto Todd-Hewitt (Oxoid) agar plates (1.5%, w/v) containing 30 g of gelatine per litre. After incubation overnight incubation at 37°C, the plates were placed at 4°C for 5 h before examination for zones of turbidity (protein hydrolysis) around the colonies. *E. faecalis* P4 was used as positive control.

Production of hemolysin

To investigate hemolysin production by the 99 LAB, the strains grown in MRS broth were streaked onto layered fresh horse blood agar plates (BioMérieux, Marcy l'Étoile, France) and grown at 37°C for 1–2 days [32]. β-hemolysis was revealed by the formation of clear zones surrounding the colonies on blood agar plates. *E. faecalis* P4 was used as positive control.

Determination of antibiotic susceptibility

Antibiotic susceptibility of the 59 enterococci was determined by overlaying antibiotic-containing disks (Oxoid) on Diagnostic Sensitivity Test Agar (Oxoid) previously seeded with approximately 1×10^5 CFU/ml of each enterococcal isolate. The antibiotics tested were ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (120 μg), nitrofurantoin (300 μg), norfloxacin (10 μg), penicillin G (10 IU), rifampicin (5 μg), teicoplanin (30 μg), tetracycline (30 μg), and vancomycin (30 μg). Inhibition zone diameters were measured after overnight incubation of the plates at 37°C. Resistance phenotypes were recorded as recommended by the Clinical and Laboratory Standards Institute [71]. *E. faecalis* CECT795 and *Staphylococcus aureus* CECT435 were used for quality control. The minimum inhibitory concentration for the 49 pre-selected LAB was determined by a broth microdilution test using e-cocci (for enterococci), and Lact-1 and Lact-2 (for non-enterococcal strains) VetMIC microplates (National Veterinary Institute, Uppsala, Sweden). The antibiotics evaluated for enterococci were ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, tetracycline, chloramphenicol, narasin, and linezolid, while for the non-enterococcal strains, the tested antibiotics were ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, chloramphenicol, neomycin, penicillin, linezolid, ciprofloxacin, rifampicin, and trimethoprim. Individual colonies were suspended in a sterile glass tube containing 5 ml saline solution (0.85% NaCl) to a turbidity of 1 in the McFarland scale (approx. 3×10^8 CFU/ml) and further diluted 1000-fold. Iso-sensitest (IST) broth (Oxoid) was used for enterococci, while LSM medium (IST:MRS, 9:1) was used for all the non-enterococcal strains except *Lactobacillus curvatus* subsp. *curvatus* BCS35, that required LSM broth supplemented with 0.03% (w/v) L-cysteine (Merck KGaA) [72]. Fifty or 100 μl of the diluted enterococcal and non-enterococcal suspensions, respectively, was added to each microplate well which was then sealed with a transparent covering tape and incubated at 37°C for 18 h (in the case of *Lb. curvatus* BCS35, the plates were incubated anaerobically at 32°C for 18 h). After incubation, MICs were established as the lowest antibiotic concentration that inhibited bacterial growth, and interpreted according to the breakpoints identified by the FEEDAP Panel and adopted by EFSA to distinguish between susceptible and resistant strains [15]. Accordingly, strains showing MICs higher than the respective breakpoint were considered as resistant. *E. faecalis* CECT795 and *S. aureus* CECT794 were used for quality control of e-cocci, and Lact-1 and Lact-2 VetMIC microplates, respectively.

Deconjugation of bile salts

The ability of the 49 pre-selected LAB to deconjugate primary and secondary bile salts was determined according to Noriega *et al.* [73]. Bile salt plates were prepared by adding 0.5% (w/v) sodium salts of taurocholate (TC) and taurodeoxycholate (TDC) (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) to MRS agar (1.5%, w/v) supplemented with 0.05% (w/v) L-cysteine (Merck KGaA, Darmstadt, Germany). Overnight liquid cultures of strains (10 μl) were spotted onto agar plates and incubated under anaerobic conditions (Anaerogen, Oxoid) at 37°C for 72 h. The presence of precipitated bile acid around the colonies (opaque halo) was considered as a positive result. A fresh fecal slurry of a healthy adult horse was used as positive control for bile salts deconjugating activities.

Degradation of mucin

The capacity of the 49 pre-selected LAB to degrade gastric mucin was determined as described by Zhou *et al.* [58]. Mucin from porcine stomach type III (Sigma-Aldrich Corp.) and agar were added to medium B without glucose at concentrations of 0.5% (w/v) and 1.5% (w/v), respectively. A volume of 10 μl of 24 h viable bacterial cultures was inoculated onto the surface of medium B. The

plates were incubated anaerobically at 37°C for 72 h, subsequently stained with 0.1% (w/v) amido black (Merck KGaA) in 3.5 M acetic acid for 30 min, and then washed with 1.2 M acetic acid (Merck KGaA). A discoloured zone around the colony was considered as a positive result. A fresh fecal slurry of a healthy adult horse was used as positive control for mucin degradation ability.

Determination of enzymatic activities

The APIZYM test (BioMérieux, Montallieu Vercieu, France) was used for determination of enzymatic activities of the 49 pre-selected LAB. Cells from cultures grown at 32°C overnight were harvested by centrifugation at 12,000 g for 2 min, resuspended in 2 ml of API Suspension Medium (BioMérieux) and adjusted to a turbidity of 5–6 in the McFarland scale (approx. 1.5-1.9 × 10^9 CFU/ml). Aliquots of 65 μl of the suspensions were added to each of the 20 reaction cupules in the APIZYM strip. The strips were incubated at 37°C for 4.5 h and the reactions were developed by addition of one drop each of the APIZYM reagents A and B. Enzymatic activities were graded from 0 to 5, and converted to nanomoles as indicated by the manufacturer´ s instructions.

PCR detection of antibiotic resistance genes

The presence of genetic determinants conferring resistance to aminoglycosides except streptomycin [*aac(6´)-Ie-aph(2´´)-Ia*], to erythromycin [*erm*(A), *erm*(B), *erm*(C) and *mef*(A/E)], to tetracycline [*tet*(K), *tet*(L) and *tet*(M)], and to lincosamides [*lnu*(A) and *lnu*(B)] was determined by PCR in the LAB strains showing antibiotic resistance by the VetMIC assay. PCR-amplifications and PCR-product visualization and analysis were performed as described above using the following primer-pairs: aacF/aacR for detection of *aac(6´)-Ie-aph(2´´)-Ia* [74], ermAI/ermAII for *erm*(A) [75,76], ermBI/ermBII for *erm*(B) [17], ermCI/ermCII for *erm*(C) [17,77], mef(A/E)I/ mef(A/E)II for *mef*(A/E) [75,76], tetKI/ tetKII for *tet*(K) [17], tetLI/tetLII for *tet*(L) [17,78], tetMI/tetMII for *tet*(M) [17,78], lnuA1/lnuA2 for *lnu*(A) [79], lnuB1/lnuB2 for *lnu*(B) [50]. *E. faecalis* C1570 was used as positive control for amplification of *erm*(C), *lnu*(A) and *tet*(K) and *E. faecalis* C1231 for *erm*(A). *E. faecium* 3Er1 (clonal complex of hospital-associated strain CC9) and *E. faecium* RC714 were used as positive controls for amplification of *aac(6´)-Ie-aph(2´´)-Ia*, *tet*(M) and *tet*(L), and for *erm*(B) and *mef*(A/E), respectively. The amplicons obtained with *mef*(A/E) and *lnu*(A) specific primers were purified by using the NucleoSpin Extract II Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and both DNA strands were sequenced at the Unidad de Genómica (Parque Científico de Madrid, Facultad de Ciencias Biológicas, Universidad Complutense de Madrid, Spain). Analysis of DNA sequences was performed with the BLAST program available at the National Center for Biotechnology Information (NCBI).

Abbreviations

LAB: Lactic Acid Bacteria; FAO: Food and Agriculture Organization of the United Nations; WHO: World Health Organization; EFSA: European Food Safety Agency; QPS: Qualified Presumption of Safety; EC: European Commission; MRS: de Man, Rogosa and Sharpe; KAA: Kanamycin, Aesculin Azide.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EMA carried out the phenotypic and genetic analyses, prepared the manuscript draft and participated in the design of the experiments. BGS carried out the isolation of the LAB strains and collaborated in the genetic studies. CA contributed to the phenotypic analyses and to prepare the manuscript draft. CC participated in the phenotypic analyses. RC collaborated in the antibiotic susceptibility tests. LMC conceived the study and, together with CH and PEH, designed the experiments, analyzed the results and revised the manuscript. All authors read and approved the final version of the manuscript.

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Author details

[1]Grupo de Seguridad y Calidad de los Alimentos por Bacterias Lácticas, Bacteriocinas y Probióticos (Grupo SEGABALBP) Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid 28040, Spain. [2]Centro de Genética e Biotecnologia, Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal. [3]Servicio de Microbiología, Hospital Universitario Ramón y Cajal, Madrid 28034, Spain.

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