

Probiotics and competitive exclusion of pathogens in shrimp aquaculture

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Abstract

Probiotics, live microorganisms that, when administered in adequate amounts, confer a health benefit on the host, offer an alternative to antibiotics and have become popular among shrimp farmers for use in the regulation of pond water quality, promotion of shrimp growth and the prevention of disease. Most shrimp probiotics are selected for testing based on their ability to competitively exclude pathogens through bacterial antagonism assays, although the mechanisms of pathogen exclusion are rarely investigated. In this review, we provide a comprehensive overview of the mechanisms of competitive exclusion (interference and exploitation competition) by species screened and subsequently identified as shrimp probiotics based on their ability to inhibit the growth of pathogenic bacteria *in vitro*. We show that the current methods used to identify potential probiotics preferentially select for interference-based competitive mechanisms and may overlook the potential of many species to be considered a probiotic. Furthermore, we show that the efficiency of a probiotic *in vivo* may be improved by considering the suitability of competitive strategies to shrimp farming conditions. We highlight important limitations and future directions for the screening and identification of probiotics in shrimp aquaculture, to aid in the development of effective and sustainable microbial management strategies.

Key words: competitive exclusion, gut microbiome, microbial ecology, probiotics, shrimp aquaculture.

Introduction

Probiotics, defined as ‘live microorganisms that, when administered in adequate amounts, confer a health benefit on the host’ (Hill *et al.*, 2014), are becoming increasingly popular antibiotic alternatives to promote growth and prevent disease in shrimp aquaculture. Whilst knowledge on their exact mechanisms of action is limited, there is strong evidence showing they are able to confer probiotic effects through the competitive exclusion of pathogenic bacteria, nutrient and enzymatic contribution to shrimp digestion, enhancement of the shrimp immune response and antiviral effects (Kumar *et al.*, 2016; Hoseinifar *et al.*, 2018; Li *et al.*, 2018; Ringø, 2020). To date, approximately 20 genera of bacteria have been shown to have a probiotic effect in shrimp, although the majority of research has focused on

Bacillus and lactic acid bacteria (LAB), such as *Lactobacillus* (Tables 1–3), due largely to their prevalence and successful application as probiotics in mammals and poultry. Probiotics can be administered orally with the feed (including bioencapsulation with live food vectors such as artemia; Immanuel, 2016), directly into the water as purified cultures or spores (Ringø, 2020), or within a fermented growth media, for example *Bacillus subtilis* E20-fermented soybean meal (Liu *et al.*, 2009, 2010; Tseng *et al.*, 2009; Tsai *et al.*, 2019; Wang *et al.*, 2019). Similarly, probiotics may be administered in combination with a complementary prebiotic, ‘a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon’ (Gibson & Roberfroid, 1995), to form treatments known as ‘synbiotics’ (Schrezenmeir & de Vrese, 2001; Li *et al.*, 2018).

Table 1 Lactic acid bacteria with a probiotic effect in shrimp aquaculture (For specific methods, see original papers)

Genus	Species	Isolated from	Host (probiotic effect for)	Inhibits (in vitro antagonism)	Method†	Challenge test (in vivo)	Source of growth inhibition	References	Probiotic effects‡
<i>Enterococcus</i>	<i>faecium</i> NRW-2	Commercial probiotics, guts of wild shrimp (<i>Fenneropenaeus chinensis</i>) and fish (<i>Chaeturichthys stigmatias</i>)	<i>Litopenaeus vannamei</i>	<i>Vibrio harveyi</i> , <i>V. parahaemolyticus</i>	Well-diffusion (CFCS)	<i>V. parahaemolyticus</i>	N/A	Sha et al. (2016a, 2016b, 2016c)	1, 3, 4, 5
	<i>faecium</i> MC13	Shrimp or fish intestines	<i>Penaeus monodon</i>	N/A	N/A	<i>V. harveyi</i> , <i>V. parahaemolyticus</i>	N/A	Swain et al. (2009)	1, 3
<i>Lactobacillus</i>	<i>acidophilus</i> 04	Homemade curd	<i>P. monodon</i>	<i>V. parahaemolyticus</i> , <i>V. cholerae</i> , <i>V. harveyi</i> and <i>V. alginolyticus</i>	Disc-diffusion (CFCS, pH neutralised)	<i>V. alginolyticus</i>	N/A, excluding the potential inhibitory effect of acid production	Sivakumar et al. (2012)	3
	<i>acidophilus</i> NCIM 2285	Culture collection	<i>F. indicus</i>	<i>V. alginolyticus</i>	Cross-streak (LC), Culture (OD) with CFCS (pH neutralised)	<i>V. alginolyticus</i>	N/A, excluding the potential inhibitory effect of acid production	Ajitha et al. (2004)	1, 3, 4, 6
	<i>acidophilus</i> RS058	Shrimp and common carp ponds	<i>L. vannamei</i>	N/A	N/A	N/A	N/A	Wang and Gu (2010)	1, 5
	<i>bulgaricus</i> E20	Digestive tracts of <i>L. vannamei</i>	<i>L. vannamei</i>	<i>V. parahaemolyticus</i>	Agar diffusion (LC)	<i>V. parahaemolyticus</i>	N/A	Roomiani et al. (2018)	2, 3, 5
	<i>bulgaricus</i> NCIM 2056, NCIM 2057	Culture collection	<i>F. indicus</i>	<i>V. alginolyticus</i>	Cross-streak (LC), Culture (OD) with CFCS (pH neutralised)	<i>V. alginolyticus</i>	N/A, excluding the potential inhibitory effect of acid production	Ajitha et al. (2004)	1, 3, 4, 6
	<i>fermentum</i> LW2	Lake water	<i>L. vannamei</i>	N/A	N/A	<i>V. alginolyticus</i>	N/A	Wang et al. (2019)	6
	<i>pentosus</i> BD6	Pigeon faeces	<i>L. vannamei</i>	N/A	N/A	<i>V. alginolyticus</i>	N/A	Wang et al. (2019)	5, 6
	<i>graminis</i>	Culture collection	<i>L. vannamei</i>	N/A	N/A	<i>V. parahaemolyticus</i>	N/A	Bernal et al. (2017)	3, 5
	<i>pentosus</i> AS13	Digestive tracts of <i>L. vannamei</i>	<i>L. vannamei</i>	<i>V. vulnificus</i> , <i>V. rotiferianus</i> and <i>V. campbellii</i>	Well-diffusion (CS)	<i>V. vulnificus</i> , <i>V. rotiferianus</i> , <i>V. campbellii</i>	N/A	Zheng and Wang (2017)	1, 3, 6
	<i>pentosus</i> HC-2	Commercial probiotics, guts of wild shrimp (<i>F. chinensis</i>) and fish (<i>C. stigmatias</i>)	<i>L. vannamei</i>	<i>V. harveyi</i> , <i>V. parahaemolyticus</i>	Well-diffusion (CFCS)	<i>V. parahaemolyticus</i>	Up-regulated expression of <i>luxS</i>	Sha et al. (2016a, 2016b, 2016c)	1, 3, 4, 5
	<i>plantarum</i>	Digestive tracts of juvenile <i>L. vannamei</i>	<i>L. vannamei</i>	<i>V. harveyi</i>	Well-diffusion (LC)	<i>V. harveyi</i> , <i>V. alginolyticus</i>	N/A	Vieira et al. (2007, 2016), Ramirez et al. (2017)	2, 3, 4, 6
	<i>plantarum</i> 7-40 (NTU102)	Korean-style cabbage pickles	<i>L. vannamei</i>	N/A	N/A	<i>V. alginolyticus</i>	N/A	Chiu et al. (2007)	3, 5
	<i>plantarum</i> MRO3.12	Digestive tracts of wild shrimp (<i>M. brevicornis</i> , <i>P. merguensis</i>) and cultured <i>L. vannamei</i>	<i>L. vannamei</i>	<i>V. harveyi</i>	Double layer (LC), Culture with CFCS (pH neutralised, catalase-treated), Co-culture	<i>V. harveyi</i>	Exclude potential antibacterial effect caused by a pH decrease and/or production of hydrogen peroxide	Kongnum and Hongpattarakere (2012)	1 – 6

Table 1 (continued)

Genus	Species	Isolated from	Host (probiotic effect for)	Inhibits (in vitro antagonism)	Method†	Challenge test (in vivo)	Source of growth inhibition	References	Probiotic effects‡
	<i>planarum</i> T8, T13	<i>L. vannamei</i> hepatopancreas	<i>L. vannamei</i>	<i>V. parahaemolyticus</i>	Well-diffusion (CFCs, pH neutralised and catalase treated)	<i>V. parahaemolyticus</i>	Exclude potential antibacterial effect caused by a pH decrease and/or production of hydrogen peroxide	Nguyen et al. (2018b)	1, 3
	<i>planarum</i> MTCC no. 1407	Culture collection	<i>M. rosenbergii</i>	<i>A. hydrophila</i> , <i>Pseudomonas fluorescens</i>	Well-diffusion (LC), Disc-diffusion (CFCs)	<i>A. hydrophila</i>	N/A	Dash et al. (2014, 2016)	1, 3, 4, 5, 6, 8
	<i>planarum</i> SGLAB01	Digestive tracts of healthy <i>L. vannamei</i>	<i>L. vannamei</i>	<i>Staphylococcus aureus</i> , <i>Aerococcus viridans</i> , <i>Bacillus megaterium</i> , <i>B. subtilis</i> , <i>V. parahaemolyticus</i> , <i>V. harveyi</i> , and <i>Escherichia coli</i>	Culture (OD) with CFCs	<i>V. parahaemolyticus</i>	N/A	Chomwong et al. (2018)	3, 5
	sp AMET1506	Curd	<i>L. vannamei</i> , <i>P. monodon</i>	<i>E. coli</i> , <i>V. cholerae</i> , <i>V. parahaemolyticus</i> , <i>Salmonella sp.</i> and <i>Shigella sp.</i>	Disc-diffusion (CFCs, pH neutralised)	<i>V. harveyi</i>	N/A, excluding the potential inhibitory effect of acid production	Karthik et al. (2014)	1, 3
	sp.	Intestine of healthy <i>L. vannamei</i>	<i>L. vannamei</i>	common pathogens in shrimp disease	Disc-diffusion (unspecified)	WSSV	N/A	Zuo et al. (2019)	1, 3, 5, 6
Lactococcus	<i>lactis</i> D1813	Gut of <i>M. japonicus</i>	<i>M. japonicus</i>	<i>V. penaeicida</i>	Culture (OD) with CFCs	<i>V. penaeicida</i>	N/A	Maeda et al. (2014)	3, 5
	<i>lactis</i> subsp. <i>cremoris</i> NCIM 2179	Culture collection	<i>F. indicus</i>	<i>V. alginolyticus</i>	Cross-streak (LC), Culture (OD) with CFCs (pH neutralised)	<i>V. alginolyticus</i>	N/A, excluding the potential inhibitory effect of acid production	Ajitha et al. (2004)	3, 4
	<i>lactis</i> subsp. <i>lactis</i>	Intestine of healthy <i>L. vannamei</i>	<i>L. vannamei</i>	<i>V. anguillarum</i> , <i>V. harveyi</i>	N/A	<i>V. anguillarum</i> , <i>V. parahaemolyticus</i>	N/A	Adel et al. (2017a), Won et al. (2020)	1–7
	<i>garvieae</i> LC149	Shrimp or fish intestines	<i>P. monodon</i>	N/A	N/A	<i>V. harveyi</i> , <i>V. parahaemolyticus</i>	N/A	Swain et al. (2009)	3 (<i>V. harveyi</i> only)
	<i>lactis</i> SGLAB02	Digestive tracts of healthy <i>L. vannamei</i>	<i>L. vannamei</i>	<i>S. aureus</i> , <i>A. viridans</i> , <i>B. megaterium</i> , <i>B. subtilis</i> , <i>V. parahaemolyticus</i> , <i>V. harveyi</i> , and <i>E. coli</i>	Culture (OD) with CFCs	<i>V. parahaemolyticus</i>	N/A	Chomwong et al. (2018)	3, 5
Pediococcus	<i>acidilactici</i> GY2	Gut of <i>M. rosenbergii</i>	<i>M. rosenbergii</i>	<i>A. hydrophila</i>	Agar diffusion (LC)	<i>A. hydrophila</i>	N/A	Miao et al. (2020)	1, 3, 5
	<i>pentosaceus</i>	Intestine of healthy <i>L. vannamei</i>	<i>L. vannamei</i>	<i>V. anguillarum</i>	N/A	<i>Vibrio anguillarum</i> , <i>V. parahaemolyticus</i>	N/A	Adel et al. (2017b), Won et al. (2020)	1, 2, 3, 5, 6, 7
<i>Streptococcus</i>	<i>cremoris</i> NCIM 2285	Culture collection	<i>F. indicus</i>	<i>V. alginolyticus</i>	N/A	<i>V. alginolyticus</i>	N/A, excluding the potential inhibitory	Ajitha et al. (2004)	1, 3, 4, 6

Table 1 (continued)

Genus	Species	Isolated from	Host (probiotic effect for)	Inhibits (<i>in vitro</i> antagonism)	Method†	Challenge test (<i>in vivo</i>)	Source of growth inhibition	References	Probiotic effects‡
	<i>phocae</i> P180	Intestine of <i>P. indicus</i>	<i>P. monodon</i>	<i>V. parahaemolyticus</i> , <i>V. vulnificus</i> , <i>V. anguillarum</i> , <i>V. fischeri</i> , <i>A. hydrophila</i> and <i>Listeria monocytogenes</i>	Cross-streak (LC), Culture (OD) with CFCS (pH neutralised) Double layer (LC), Well diffusion (unspecified)	<i>V. harveyi</i>	effect of acid production Detection of bacteriocin activity (Tricine SDS-PAGE electrophoresis). Bacteriocin (phocaein P180) characterised by Satish Kumar and Arul (2009)	Swain et al. (2009), Kanmani et al. (2010)	1, 3

†Method: Double layer (CS, LC); Probiotic cultured on agar medium. Colonies killed by exposure to chloroform (culture supernatant; CS) or left and used as live cultures (LC). Pathogen cultured in liquid media, mixed (seeded) with soft agar and poured over the probiotic plate. Incubated and inhibition of pathogen growth recorded. Cross-streak (CS, LC): Probiotic and pathogen streaked perpendicular to each other on plates (LC). Alternatively, probiotic streaked on plate, incubated and growth scraped. Remaining bacteria killed by exposure to chloroform (CS). Pathogenic strain streaked perpendicular to probiotic. Growth inhibition observed as linear clear zones. Co-culture (LC): Both probiotic and pathogen cultured together in liquid media (broth). Samples withdrawn, serially diluted and plated to determine viable cell counts (growth enumerated on agar). Culture (CS, CFCS): Pathogen cultured in liquid media (broth) with probiotic CS or cell free culture supernatant (CFCS). Growth and bacteriocin activity measured using optical density (OD) or samples withdrawn, serially diluted and plated to determine viable pathogen count. Disc-diffusion (CFCS, LC): Discs impregnated with CFCS or LC of probiotics positioned on top of lawn cultures of pathogenic bacteria. Incubated and then clear zones of inhibition around the disc measured. Well-diffusion (CFCS, LC): Pathogen suspensions spread evenly over agar plate (lawn culture). Wells punched into solidified agar and filled with CFCS or LC of probiotic. Incubated and (clear) zones of inhibition around the wells measured. Agar diffusion (LC); pathogen spread onto agar plate (lawn culture). Small amounts of probiotic LC smeared or spotted over lawn culture. Zones of inhibition surrounding the plaques of probiotic culture measured. Co-culture (F): Both probiotic and pathogen (expressing green fluorescent protein (GFP)) cultured together in liquid media (broth). Fluorescence (F) signal generated by GFP-expressing cells used to quantify the bacterial growth.

‡Probiotic effect: (1) Increased growth (including weight gain), (2) increased survival (no pathogen challenge), (3) increased survival (pathogen challenge), (4) decreased pathogen counts (including presumptive *Vibrio* sp.), (5) immunomodulatory effect, (6) increased in digestive efficiency (including digestive enzyme activity, feed conversion ratio, feed efficiency or protein efficiency ratio), (7) morphological changes in intestines (including increased villi height and number of folds/revines), and (8) improved water quality (including reduction in presumptive pathogen counts).

Table 2 *Bacillus* species with a probiotic effect in shrimp aquaculture (For specific methods see original papers)

Genus Species	Isolated from	Host (probiotic effect for)	Inhibits (<i>in vitro</i> antagonism)	Method†	Challenge test (<i>in vivo</i>)	Source of growth inhibition	Reference	Probiotic effects‡
<i>Bacillus subtilis</i> MTCC 121	Culture collection	<i>Macrobrachium rosenbergii</i>	N/A	N/A	N/A	N/A	Karthik <i>et al.</i> (2018)	1, 2, 4, 6
<i>amyloliquefaciens</i>	Digestive tracts of adult <i>Litopenaeus vannamei</i>	<i>L. vannamei</i>	<i>Vibrio campbellii</i> , <i>V. vulnificus</i> , <i>V. parahaemolyticus</i> , <i>V. harveyi</i>	Double layer (CS), Well-diffusion (LC)	N/A	N/A	Luis-Villaseñor <i>et al.</i> (2011)	1, 2
<i>aquamaris</i> SH6	Gastrointestinal tract (GIT) of wild shrimp	<i>L. vannamei</i>	N/A	N/A	N/A	N/A	Ngo <i>et al.</i> (2016), Nguyen <i>et al.</i> (2018a, 2020)	1, 5, 10
<i>aryabhattai</i> TBRC8450	Sediment from shrimp farm	<i>L. vannamei</i>	<i>V. harveyi</i> , <i>V. parahaemolyticus</i>	Agar diffusion (LC)	<i>V. harveyi</i>	N/A	Tepaamorndech <i>et al.</i> (2019)	3, 4, 5, 12
<i>cereus</i>	Gut of healthy <i>M. rosenbergii</i>	<i>M. rosenbergii</i>	N/A	N/A	<i>A. hydrophila</i>	N/A	Wee <i>et al.</i> (2018)	1, 5
<i>cereus</i>	Seawater, sediment and gut content of healthy fish (<i>Lates calcarifer</i>)	<i>P. monodon</i>	<i>V. harveyi</i> , <i>V. vulnificus</i> , <i>Vibrio</i> spp.	Well diffusion (LC)	<i>V. harveyi</i> , <i>Vibrio</i> spp.	N/A	Ravi <i>et al.</i> (2007)	3
<i>cereus</i>	GIT of <i>L. vannamei</i>	<i>L. vannamei</i>	<i>V. harveyi</i>	Disc-diffusion (LC)	<i>V. harveyi</i>	Detection of bacteriocin activity (SDS-PAGE electrophoresis)	Masitoh <i>et al.</i> (2016)	3, 5
<i>cereus</i>	Intestine of juvenile <i>L. vannamei</i>	<i>L. vannamei</i>	N/A	N/A	<i>V. parahaemolyticus</i> , <i>V. alginolyticus</i>	N/A	Vidal <i>et al.</i> (2018)	4, 11
<i>cereus</i>	Gut of wild <i>P. monodon</i>	<i>P. monodon</i>	<i>V. harveyi</i> , <i>Staphylococcus aureus</i> , <i>Escherichia coli</i> , <i>Bacillus subtilis</i> , <i>Aeromonas hydrophila</i>	Well-diffusion (CFCS)	N/A	N/A	Chandran <i>et al.</i> (2014, 2017)	1, 2, 5, 6
<i>coagulans</i>	Gut of wild <i>P. monodon</i>	<i>P. monodon</i>	<i>V. parahaemolyticus</i>	N/A	<i>V. parahaemolyticus</i>	N/A	Raghu <i>et al.</i> (2016)	5
<i>coagulans</i> (ATCC 7050)	Culture collection	<i>L. vannamei</i>	N/A	N/A	<i>V. parahaemolyticus</i>	N/A	Amoah <i>et al.</i> (2019)	1, 3, 5, 6, 7, 12
<i>coagulans</i> (MTCC 2302)	Culture collection	<i>M. rosenbergii</i>	N/A	N/A	N/A	N/A	Karthik <i>et al.</i> (2018)	1, 2, 4, 6
<i>coagulans</i> NJ105	Shrimp and common carp ponds	<i>L. vannamei</i>	N/A	N/A	N/A	N/A	Wang and Gu (2010), Wang <i>et al.</i> (2012)	1, 2, 5, 6
<i>coagulans</i> SC8168	Shrimp pond sediment	<i>L. vannamei</i>	N/A	N/A	N/A	N/A	Zhou <i>et al.</i> (2009)	2, 6
<i>endophyticus</i>	Digestive tracts of adult <i>L. vannamei</i>	<i>L. vannamei</i>	<i>V. parahaemolyticus</i> , <i>V. harveyi</i>	Double layer (CS), Well-diffusion (LC)	N/A	N/A	Luis-Villaseñor <i>et al.</i> (2011)	1, 2
<i>frumus</i>	Gut of wild <i>P. monodon</i>	<i>P. monodon</i>	<i>V. parahaemolyticus</i>	N/A	<i>V. parahaemolyticus</i>	N/A	Raghu <i>et al.</i> (2016)	5
<i>flexus</i> LD-1	Pond waters/sediments	<i>L. vannamei</i>	<i>V. parahaemolyticus</i> , <i>V. alginolyticus</i>	Double layer (LC)	<i>V. harveyi</i>	N/A	Cai <i>et al.</i> (2019)	2, 3, 4, 5, 6, 8, 9
<i>fusiformis</i>	Fermented solution	<i>L. vannamei</i> , <i>P. monodon</i>	<i>Streptococcus iniae</i> , <i>Photobacterium damselae</i> subsp. <i>piscicida</i>	Co-culture (LC)	N/A	N/A	Guo <i>et al.</i> (2009)	2
<i>licheniformis</i>	Shrimp pond sediment	<i>L. vannamei</i>	N/A	N/A	N/A	N/A. Selected for its ability to secrete extracellular	Li <i>et al.</i> (2007)	4, 5

Table 2 (continued)

Genus	Species	Isolated from	Host (probiotic effect for)	Inhibits (in vitro antagonism)	Method†	Challenge test (in vivo)	Source of growth inhibition	Reference	Probiotic effects‡
<i>Licheniformis</i>		Culture collection	<i>Macrobrachium rosenbergii</i> <i>L. vannamei</i>	N/A	N/A	<i>V. alginolyticus</i>	Macromolecule digesting enzymes	Kumar et al. (2013)	1, 5, 3
<i>Licheniformis</i>	ClGBC-232	<i>L. vannamei</i> intestine	<i>V. harveyi</i>	<i>V. harveyi</i>	Double layer (CS)	N/A	N/A	Franco et al. (2017)	1, 5, 9
<i>Licheniformis</i>	DAH81	Gut of <i>Fenneropenaeus indicus</i>	<i>F. indicus</i>	<i>V. parahaemolyticus</i>	N-acetylated homoserine lactone (AHL)-lactonase bioassay, biofilm development assay	N/A	Production of AHL-lactonase, shown to inhibit <i>V. parahaemolyticus</i> biofilm formation	Vinoj et al. (2014)	3, 4
<i>Licheniformis</i>	LS-1 megaterium	Pond waters/sediments Mangrove sediment	<i>L. vannamei</i> <i>P. monodon</i>	<i>V. harveyi</i> , <i>V. vulnificus</i>	Double layer (LC)	<i>V. harveyi</i> N/A	N/A	Cai et al. (2019)	1, 2, 3, 4, 5, 6, 8, 9
<i>megaterium</i>		N/A	<i>L. vannamei</i>	N/A	N/A	N/A	N/A	Aftabuddin et al. (2013)	1, 8
OJ		<i>L. vannamei</i> intestine	<i>L. vannamei</i>	N/A	N/A	N/A	N/A	Olmos et al. (2011)	9
PC465		Intestine of <i>F. chinensis</i>	<i>L. vannamei</i>	N/A	N/A	WSSV	N/A	Li et al. (2009)	3, 4, 5
<i>pumilus</i>		Culture collection	<i>P. monodon</i>	N/A	N/A	WSSV	N/A	Chai et al. (2016)	1, 2, 3, 5, 6, 7, 12
sp.		N/A	<i>L. vannamei</i>	N/A	N/A	N/A	N/A	Banerjee et al. (2010)	1, 2, 4, 8
sp. MIA1.1, MIA2.1		mucus of tilapia (<i>Oreochromis</i> sp.)	<i>P. monodon</i>	<i>V. parahaemolyticus</i>	Agar diffusion (LC), Co-culture (LC)	N/A	N/A	Lin et al. (2004)	6
sp. Mk22		Salt-pan sediment	<i>P. monodon</i>	<i>V. parahaemolyticus</i> , <i>V. harveyi</i>	Well-diffusion (unspecified)	<i>V. parahaemolyticus</i>	N/A	Doroteo et al. (2018)	2, 9
sp. NFMI-C		Open cultures of the microalgae <i>Chaetoceros muelleri</i>	<i>M. rosenbergii</i>	N/A	N/A	<i>V. campbellii</i>	Inhibition of acylhomoserine lactones (AHL) quorum sensing by AHL degradation	Ashokkumar et al. (2016)	1, 2, 3, 4, 6
sp. NL110		<i>M. rosenbergii</i> larvae	<i>M. rosenbergii</i>	<i>A. hydrophila</i> , <i>V. parahaemolyticus</i> , <i>V. vulnificus</i> , <i>V. harveyi</i> , <i>E. coli</i> , <i>Salmonella Newport</i> , <i>S. typhi</i>	Well-diffusion (LC), Cross-streak (CS)	N/A	N/A	Mayavu (2014), Ashokkumar et al. (2015)	3
sp. P11		GIT of <i>P. monodon</i> broodstock	<i>P. monodon</i> , <i>L. vannamei</i>	<i>V. harveyi</i> , <i>E. coli</i>	Agar diffusion (LC)	<i>V. harveyi</i>	18 antibiotics undetected in CFCS (screening test kit).	Rahiman et al. (2010)	1, 2, 5, 8
sp. P64		Homogenised wild <i>L. vannamei</i>	<i>L. vannamei</i>	<i>V. harveyi</i>	Agar diffusion (LC)	<i>V. harveyi</i>	N/A	Utiswannakul et al. (2011), Sapcharoen and Rengpipat (2013)	1, 2, 3, 6
sp. S11		GIT of <i>P. monodon</i> broodstock	<i>P. monodon</i> , <i>L. vannamei</i>	<i>V. harveyi</i> , <i>V. parahaemolyticus</i>	Agar diffusion (LC)	<i>V. harveyi</i>	18 antibiotics undetected in CFCS (screening test kit).	Gullian et al. (2004)	5, 1, 3
<i>subtilis</i>		Juvenile <i>M. rosenbergii</i> gut	<i>M. rosenbergii</i>	<i>A. hydrophila</i> , <i>V. parahaemolyticus</i>	Double layer (CS)	<i>A. hydrophila</i>	N/A	Rengpipat et al. (1998, 2000, 2003), Povedchagun et al. (2011), Sapcharoen and Rengpipat (2013)	1, 2, 3, 5
								Keysami et al. (2012),	1, 2, 3, 6

Table 2 (continued)

Genus	Species	Isolated from	Host (probiotic effect for)	Inhibits (in vitro antagonism)	Method†	Challenge test (in vivo)	Source of growth inhibition	Reference	Probiotic effects‡
<i>subtilis</i>		N/A	<i>L. vannamei</i>	N/A	N/A	N/A	N/A	Keysami and Mohammadpour (2013) Oimos et al. (2011)	1, 9
<i>subtilis</i>	(IPA-S51)	<i>L. vannamei</i> hepatopancreas/stomach	<i>L. vannamei</i>	<i>V. alginolyticus</i>	Well diffusion (CFCS, LC)	N/A	N/A	Interminense et al. (2018)	1
<i>subtilis</i>	BT23	Shrimp culture ponds	<i>P. monodon</i>	<i>V. harveyi</i> , <i>V. anguillarum</i> , <i>V. vulnificus</i> , <i>V. damsela</i>	Well-diffusion (CFCS), Co-culture (LC with <i>V. harveyi</i>). Culture with CFCS	<i>V. harveyi</i>	N/A	Vaseeharan and Ramasamy (2003)	3
<i>subtilis</i>	E20	Natto (fermented soybeans)	<i>L. vannamei</i>	<i>A. hydrophila</i>	Disc-diffusion (CFCS) (<i>V. harveyi</i> , OD)	<i>V. alginolyticus</i>	Two antimicrobial peptides with antimicrobial activity against <i>V. alginolyticus</i> and <i>V. parahaemolyticus</i> identified using an RP-nano-ultrapure liquid chromatography electrospray ionization-tandem mass spectroscopic analysis (Cheng et al., 2017; Cheng et al., 2020)	Liu et al. (2009, 2010), Tseng et al. (2009), Tsai et al. (2019), Wang et al. (2019)	1, 2, 3, 5, 6, 9
<i>subtilis</i>	S12	<i>L. vannamei</i> digestive content	<i>L. vannamei</i>	<i>A. hydrophila</i> , <i>Aeromonas sobria</i> , <i>Aeromonas caviae</i> , <i>Aeromonas hydrophila</i> , <i>V. anguillarum</i> , <i>V. vulnificus</i> , <i>V. alginolyticus</i> , <i>V. harveyi</i> , <i>V. parahaemolyticus</i> , <i>V. parahaemolyticus</i> , <i>V. vulnificus</i>	Well diffusion (LC, CFCS)	<i>V. harveyi</i>	N/A	Liu et al. (2014)	1, 2, 3, 5
<i>subtilis</i>	SH23	Gut of white-leg shrimp	<i>L. vannamei</i>	<i>V. parahaemolyticus</i>	Well-diffusion (unspecified)	N/A	N/A	Nguyen et al. (2020)	5
<i>subtilis</i>	UTM 126	GIT of adult <i>L. vannamei</i>	<i>L. vannamei</i>	<i>V. alginolyticus</i> , <i>V. parahaemolyticus</i> , <i>V. harveyi</i>	Disc-diffusion (CFCS (pH neutralised)), Well-diffusion (CFCS)	<i>V. harveyi</i> , <i>V. parahaemolyticus</i>	N/A, excluding the potential inhibitory effect of acid production	Balcázar and Rojas-Luna (2007), Balcázar et al. (2007)	3, 1, 6, 2
<i>subtilis</i>	WB60	Intestine of juvenile Japanese eel	<i>L. vannamei</i>	N/A	Agar diffusion (LC)	<i>V. parahaemolyticus</i>	N/A	Won et al. (2020)	1, 6, 3, 5, 7
<i>tequiensis</i>		Digestive tracts of adult <i>L. vannamei</i>	<i>L. vannamei</i>	<i>V. campbellii</i> , <i>V. vulnificus</i> , <i>V. parahaemolyticus</i> , <i>V. harveyi</i>	Double layer (CS), Well-diffusion (LC)	N/A	N/A	Luis-Vilaseñor et al. (2011)	1, 2
<i>thuringiensis</i>		GIT of <i>L. vannamei</i>	<i>L. vannamei</i>	<i>V. harveyi</i>	Disc-diffusion (LC)	<i>V. harveyi</i>	Detection of bacteriocin activity (SDS-PAGE electrophoresis)	Masitoh et al. (2016)	3, 5
<i>thuringiensis</i>	G5-8-3T02	<i>P. monodon</i> intestine	<i>P. monodon</i>	<i>V. mimicus</i>		<i>V. mimicus</i>			1, 3

Table 2 (continued)

Genus Species	Isolated from	Host (probiotic effect for)	Inhibits (<i>in vitro</i> antagonism)	Method†	Challenge test (<i>in vivo</i>)	Source of growth inhibition	Reference	Probiotic effects‡
<i>vireti</i> 01	<i>M. rosenbergii</i> intestine	<i>M. rosenbergii</i>	<i>Pseudomonas aeruginosa</i>	Well-diffusion (LC, CFCS)	<i>Pseudomonas aeruginosa</i>	Antimicrobial activity of CFCS not lost after treatment with enzymes (pepsin, catalase, lipase and α -amylase) and surfactants (Sodium dodecyl sulphate and Tween 80). Heat (37°C – 121°C) and pH (2-9) stable. Gas Chromatography-Mass Spectrometry (GC-MS) of CFCS revealed 15 bioactive compounds. The main compound produced was 2, 6, 10-Trimethyltridecane. GC-MS analysis revealed major peaks for pyrrolo [1,2- <i>a</i>]pyrazine-1,4-dione, hexahydro- and 9-octadecanamide (Z) and the presence of 2, 5-piperazinedione and 3, 6-bis (2-methylpropyl)	Anyanwu and Ariole (2019), Ariol and Anyanwu (2020)	

†Method: Double layer (CS, LC): Probiotic cultured on agar medium. Colonies killed by exposure to chloroform (culture supernatant; CS) or left and used as live cultures (LC). Pathogen cultured in liquid media, mixed (seeded) with soft agar and poured over the probiotic plate. Incubated and inhibition of pathogen growth recorded. Cross-streak (CS, LC): Probiotic and pathogen streaked perpendicular to each other on plates (LC). Alternatively, probiotic streaked on plate, incubated and growth scraped. Remaining bacteria killed by exposure to chloroform (CS). Pathogenic strain streaked perpendicular to probiotic. Growth inhibition observed as linear clear zones. Co-culture (LC): Both probiotic and pathogen cultured together in liquid media (broth). Samples withdrawn, serially diluted and plated to determine viable cell counts (growth enumerated on agar). Culture (CS, CFCS): Pathogen cultured in liquid media (broth) with probiotic CS or cell free culture supernatant (CFCS). Growth and bacteriolytic activity measured using optical density (OD) or samples withdrawn, serially diluted and plated to determine viable pathogen count. Disc-diffusion (CFCS, LC): Discs impregnated with CFCS or LC of probiotics positioned on top of lawn cultures of pathogenic bacteria. Incubated and then clear zones of inhibition around the disc measured. Well-diffusion (CFCS, LC): Pathogen suspensions spread evenly over agar plate (lawn culture). Wells punched into solidified agar and filled with CFCS or LC of probiotic. Incubated and (clear) zones of inhibition around the wells measured. Agar diffusion (LC); pathogen spread onto agar plate (lawn culture). Small amounts of probiotic LC smeared or spotted over lawn culture. Zones of inhibition surrounding the plaques of probiotic culture measured. Co-culture (F): Both probiotic and pathogen (expressing green fluorescent protein (GFP)) cultured together in liquid media (broth). Fluorescence (F) signal generated by GFP-expressing cells used to quantify the bacterial growth.

‡Probiotic effect: (1) increased growth (including weight gain), (2) increased survival (no pathogen challenge), (3) increased survival (pathogen challenge), (4) decreased pathogen counts (including presumptive *Vibrio* sp.), (5) immunomodulatory effect, (6) increased in digestive efficiency (including digestive enzyme activity, feed conversion ratio, feed efficiency or protein efficiency ratio), (7) morphological changes in intestines (including increased villi height and number of folds/revines), (8) improved water quality (including reduction in presumptive pathogen counts), (9) increased survival (stress test), (10) improved pigmentation, (11) prevention of weight loss during pathogen challenge and (12) improved antioxidant activity.

Table 3 Bacterial species with a probiotic effect in shrimp aquaculture (For specific methods see original papers)

Genus	Species	Isolated from	Host (probiotic effect for)	Inhibits (in vitro antagonist)	Method†	Challenge test (in vivo)	Source of growth inhibition	References	Probiotic effects‡
<i>Alteromonas</i> <i>Arthrobacter</i>	sp. BY-9 endensis	Coastal waters	<i>Penaeus monodon</i>	<i>Vibrio harveyi</i>	N/A	N/A	N/A	Haryanti et al. (2001)	1, 2, 4
	sp. CW9 XE-7	Water column of <i>L. vannamei</i> Guts of <i>L. vannamei</i> <i>P. chinensis</i> culture water	<i>L. vannamei</i> <i>L. vannamei</i> <i>P. chinensis</i>	N/A N/A <i>V. parahaemolyticus</i> , <i>V. anguillarum</i> and <i>V. nereis</i>	N/A N/A Agar diffusion technique (LC)	N/A N/A <i>V. parahaemolyticus</i> , <i>V. anguillarum</i> and <i>V. nereis</i> (combined)	N/A N/A N/A	Xue et al. (2016) Xia et al. (2014) Li et al. (2006)	2 1, 2, 5 3, 8
	XE-7 <i>butyricum</i>	<i>L. vannamei</i> culture water Zhongke Biotic Co., Ltd, China	<i>L. vannamei</i> <i>Marsupenaeus japonicus</i>	N/A N/A	N/A N/A	N/A <i>V. parahaemolyticus</i>	N/A N/A	Li et al. (2008) Duan et al. (2017)	3, 4, 5 1, 7, 9
<i>Clostridium</i>	<i>butyricum</i>	Jiangsu Suwei Microbiology Research Co., Ltd. (China)	<i>M. rosenbergii</i>	N/A	N/A	N/A	N/A	Tadese et al. (2020)	1
	<i>butyricum</i>	Intestinal tract of <i>M. rosenbergii</i>	<i>M. rosenbergii</i>	<i>V. harveyi</i>	Co-culture (LC)	<i>V. harveyi</i>	N/A	Sumon et al. (2018)	1, 4, 6
<i>Enterobacter</i>	<i>butyricum</i> CBG01	Culture collection	<i>L. vannamei</i>	N/A	N/A	<i>V. parahaemolyticus</i>	N/A	Li et al. (2019a,b)	1, 3, 5, 6, 7
	<i>hormaechei</i>	Intestine of healthy <i>L. vannamei</i>	<i>L. vannamei</i>	common pathogens in shrimp disease (unspecified)	Disc-diffusion (method unspecified)	WSSV	N/A	Zuo et al. (2019)	1, 3, 5, 6
<i>Microbacterium</i> <i>Paenibacillus</i>	<i>aquimaris</i> <i>polymyxa</i> ATCC 842 spp.	Water column of <i>L. vannamei</i> Culture collection	<i>L. vannamei</i> <i>L. vannamei</i>	N/A N/A	N/A N/A	N/A <i>V. parahaemolyticus</i>	N/A N/A	Xue et al. (2016) Amoah et al. (2020)	2 1, 2, 3, 4, 5, 6, 7, 10
	<i>gallaeciensis</i> sp. CDA22, sp. CDM8	Seawater, sediment and gut content of healthy fish (<i>Lates calcarifer</i>) Water column of <i>L. vannamei</i> Hindgut of healthy <i>L. vannamei</i>	<i>P. monodon</i> <i>L. vannamei</i> <i>L. vannamei</i>	<i>V. harveyi</i> , <i>V. vulnificus</i> , <i>Vibrio</i> spp.	Well diffusion (LC)	N/A	N/A	Xue et al. (2016) Wang et al. (2018)	3 2, 3, 4
<i>Phaeobacter</i> <i>Pseudovibrio</i>	sp. NC201	Marine (intertidal)	<i>Litopenaeus stylirostris</i>	<i>V. nigripulchritudo</i> , <i>V. harveyi</i>	Agar diffusion (method unspecified), Co-culture (f)	<i>V. nigripulchritudo</i>	N/A	Pham et al. (2014), Sorieul et al. (2018)	2, 3, 5, 9
	<i>aestumaria</i> SLN22 sp. PS-102	Gastrointestinal tract (GIT) of adult <i>L. vannamei</i> Brackish water	<i>L. vannamei</i>	<i>V. parahaemolyticus</i>	Well-diffusion (CFCS)	<i>V. parahaemolyticus</i>	N/A	Balcázar et al. (2007)	1, 2, 3, 6
<i>Pseudomonas</i>	sp. W3	Intensive shrimp cultivation pond water	<i>L. vannamei</i>	<i>V. harveyi</i> , <i>V. vulnificus</i> , <i>V. parahaemolyticus</i> , <i>V. alginolyticus</i> , <i>V. fluvialis</i> , <i>Aeromonas</i> spp	Disc-diffusion (CFCS)	N/A	Antimicrobial properties of CFCS not affected after treatment with proteinase K, pronase, α -chymo- trypsin, trypsin, lysozyme, lipase, and α -amylase. Heat stable (some activity lost). Produced siderophores belonging to a catechol group.	Vijayan et al. (2006)	N/A
	sp. 17-1 <i>palustris</i> GH642	GIT of healthy <i>L. vannamei</i> Shrimp and common carp ponds	<i>L. vannamei</i> <i>L. vannamei</i>	<i>V. harveyi</i> , <i>V. parahaemolyticus</i> , <i>V. cholerae</i>	Well diffusion (LS, CFCS), Culture (CS, CFCS with live and heat-killed <i>V. harveyi</i> : OD)	<i>V. harveyi</i>	Produces 2-Heptyl-4-quinolone (HHQ). Small molecular weight, heat stable, pH resistant and mostly tolerant to a variety of enzymes.	Rattanachay et al. (2007, 2010, 2011)	3
<i>Psychrobacter</i> <i>Rhodopsseudomonas</i>	sp. 17-1 <i>palustris</i> GH642	GIT of healthy <i>L. vannamei</i> Shrimp and common carp ponds	<i>L. vannamei</i> <i>L. vannamei</i>	<i>V. harveyi</i> , <i>Aeromonas</i> sp.	Double layer (CS)	<i>Aeromonas</i> sp.	N/A	Franco et al. (2016)	1, 3, 5, 6, 9
				N/A	N/A	N/A	N/A	Wang and Gu (2010)	1, 5

Table 3 (continued)

Genus	Species	Isolated from	Host (probiotic effect for)	Inhibits (in vitro antagonism)	Method†	Challenge test (in vivo)	Source of growth inhibition	References	Probiotic effect‡
Roseobacter	<i>gallaecensis</i> SLV03	GIT of adult <i>L. vannamei</i>	<i>L. vannamei</i>	<i>V. parahaemolyticus</i>	Well-diffusion (CFCS)	<i>V. parahaemolyticus</i>	N/A	Balcázar et al. (2007)	1, 2, 3, 6
	<i>fradiae</i>	Mangrove sediment	<i>P. monodon</i>	N/A	N/A	N/A	N/A	Afabbuddin et al. (2013)	1, 8
Streptomyces	<i>rubrolaevendulae</i> M56	Coastal sediment	<i>P. monodon</i>	<i>V. harveyi</i> , <i>V. parahaemolyticus</i> , <i>V. alginolyticus</i> , <i>V. fluvialis</i>	Co-culture (LC)	N/A	N/A	Augustine et al. (2016)	2, 4
	sp. CLS-28	Coastal sediment Shrimp pond sediment	<i>P. monodon</i> <i>P. monodon</i>	N/A <i>V. harveyi</i> , <i>V. parahaemolyticus</i> , <i>V. proteolyticus</i> , <i>V. anguillarum</i> , <i>V. alginolyticus</i>	N/A Cross-streak (CS)	N/A <i>V. harveyi</i>	N/A N/A	Das et al. (2006) Das et al. (2010)	1, 4, 8 1, 2, 3, 4, 6
Vibrio	sp. CLS-39, sp. CLS-45	Shrimp pond sediment	<i>P. monodon</i>	<i>V. harveyi</i> , <i>V. parahaemolyticus</i> , <i>V. proteolyticus</i> , <i>V. anguillarum</i> , <i>V. alginolyticus</i>	Cross-streak (CS)	<i>V. harveyi</i>	N/A	Das et al. (2010)	1, 2, 3
	sp. N7	Marine sediment	<i>L. vannamei</i>	<i>V. harveyi</i> , <i>V. vulnificus</i> , <i>V. parahaemolyticus</i>	Well-diffusion (LC)	<i>V. parahaemolyticus</i>	N/A	Bernal et al. (2015, 2017)	1, 4
Vibrio	sp. RL8	Marine sediment	<i>L. vannamei</i>	<i>V. harveyi</i> , <i>V. vulnificus</i> , <i>V. parahaemolyticus</i>	Well-diffusion (LC)	<i>V. parahaemolyticus</i>	N/A	Bernal et al. (2015, 2017)	1, 3, 4
	<i>alginolyticus</i> UTM 102	GIT of adult <i>L. vannamei</i>	<i>L. vannamei</i>	<i>V. parahaemolyticus</i> , <i>V. parahaemolyticus</i>	Well-diffusion (CFCS)	<i>V. parahaemolyticus</i>	N/A	Balcázar et al. (2007)	1, 3, 6
Vibrio	Sp.	Marine (intertidal)	<i>L. stylostris</i>	<i>V. nigripurpuritudo</i> , <i>V. harveyi</i>	Diffusion agar assay (method unspecified), Co-culture (F)	N/A	N/A	Pham et al. (2014)	2, 5
	sp. NE17	<i>M. rosenbergii</i> eggs	<i>M. rosenbergii</i>	<i>A. hydrophila</i> , <i>V. parahaemolyticus</i> , <i>V. vulnificus</i> , <i>E. coli</i> , <i>S. newport</i>	Well-diffusion (LC), Cross-streak (CS)	N/A	N/A	Rahiman et al. (2010)	1, 2, 5, 8
Vibrio	sp. P62, sp. P63	Homogenised wild <i>L. vannamei</i>	<i>L. vannamei</i>	<i>V. harveyi</i>	Agar diffusion (LC)	<i>V. harveyi</i>	N/A	Gullian et al. (2004)	1, 3

†Method: Double layer (CS, LC); Probiotic cultured on agar medium. Colonies killed by exposure to chloroform (culture supernatant; CS) or left and used as live cultures (LC). Pathogen cultured in liquid media, mixed (seeded) with soft agar and poured over the probiotic plate. Incubated and inhibition of pathogen growth recorded. Cross-streak (CS, LC); Probiotic and pathogen streaked perpendicular to each other on plates (LC). Alternatively, probiotic streaked on plate, incubated and growth scraped. Remaining bacteria killed by exposure to chloroform (CS). Pathogenic strain streaked perpendicular to probiotic. Growth inhibition observed as linear clear zones. Co-culture (LC): Both probiotic and pathogen cultured together in liquid media (broth). Samples withdrawn, serially diluted and plated to determine viable cell counts (growth enumerated on agar). Culture (CS, CFCS): Pathogen cultured in liquid media (broth) with probiotic CS or cell free culture supernatant (CFCS). Growth and bacteriolytic activity measured using optical density (OD) or samples withdrawn, serially diluted and plated to determine viable pathogen count. Disc-diffusion (CFCS, LC): Discs impregnated with CFCS or LC of probiotics positioned on top of lawn cultures of pathogenic bacteria. Incubated and then clear zones of inhibition around the disc measured. Well-diffusion (CFCS, LC): Pathogen suspensions spread evenly over agar plate (lawn culture). Wells punched into solidified agar and filled with CFCS or LC of probiotic. Incubated and (clear) zones of inhibition around the wells measured. Agar diffusion (LC); pathogen spread onto agar plate (lawn culture). Small amounts of probiotic LC smeared or spotted over lawn culture. Zones of inhibition surrounding the plaques of probiotic culture measured. Co-culture (F): Both probiotic and pathogen (expressing green fluorescent protein (GFP)) cultured together in liquid media (broth). Fluorescence (F) signal generated by GFP-expressing cells used to quantify the bacterial growth.

‡Probiotic effect: (1) Increased growth (including weight gain), (2) increased survival (no pathogen challenge), (3) increased survival (pathogen challenge), (4) decreased pathogen counts (including presumptive *Vibrio* sp.), (5) immunomodulatory effect, (6) increased in digestive efficiency (including digestive enzyme activity, feed conversion ratio, feed efficiency or protein efficiency ratio), (7) morphological changes in intestines (including increased villi height and number of folds/revines), (8) improved water quality (including reduction in presumptive pathogen counts), (9) increased survival (stress test) and (10) improved antioxidant activity.

Probiotic species are often isolated from the intestines of shrimp and the surrounding water or sediment of their environment (examples are shown in Tables 1–3). However, they have also been isolated from fruit waste filtrates (Nurliana *et al.*, 2020), curd (Karthik *et al.*, 2014), fermented soybeans ‘Nattō’ (Liu *et al.*, 2010), fermented pickles (Zokaeifar *et al.*, 2012a,b) and the intestines of other species. For example, in the case of the latter, shrimp probiotic *Lactobacillus* species have been isolated from the digestive tracts of chickens (Phianphak *et al.*, 1999) and fish (Sha *et al.*, 2016a; Doroteo *et al.*, 2018). Commercial probiotic treatments, which largely contain LAB and *Bacillus* spp., have also been tested for their probiotic effects in shrimp (Ringø, 2020). Lack of strict regulations regarding commercial probiotics intended for aquaculture in some regions, however, has raised concerns regarding the reliability of the labelling of such treatments. As an example to illustrate this, Vargas-Albores *et al.* (2016) detected both additional and different bacterial species in the commercial probiotic Eco-AQUAPROTEC (Eco Technology Solutions, Australia) to those reported for the product by the manufacturer. Furthermore, it is also the case that commercial probiotics often contain more than one microbial species, as well as feed additives such crude protein and fibre, and enzymes such as proteases, amylases, cellulase, xylanase and beta-glucanases. This, in turn, often makes it difficult to attribute probiotic effects to any individual species or component of the formulation to be able to characterise their underlying effect mechanisms. In this review, we focus on shrimp probiotic treatments containing a single species, including those isolated from commercial products to better understand competitive exclusion mechanisms in shrimp probiotics. For an extensive list of commercial and multi-species probiotics that have been investigated, we refer the reader to the recent paper by Ringø (2020).

Prior to *in vivo* testing, a number of criteria are considered by researchers during the screening and selection of potential probiotic species in shrimp aquaculture. For example, ease of culture, biosafety (including haemolytic activity and antibiotic susceptibility) and their ability to produce extracellular enzymes and competitively exclude pathogens. However, after isolation, potential probiotics are more often than not selected for based on the competitive exclusion principle (that species competing for the same limited resources cannot co-exist; Volterra, 1928; Gause, 1932; Hardin, 1960; Levin, 1970), through bacterial antagonism assays, in which pathogens are exposed directly (co-culture) or indirectly (extracellular products) to candidate bacteria (Tables 1–3). If they show a significant inhibitory effect against the pathogenic bacteria and are presumed to be safe to the host organism (the presence of safety-related virulence factors such as haemolytic activity (Chang *et al.*, 2000) and chitinase production (Defoirdt

et al., 2010; Frederiksen *et al.*, 2013) are often not determined prior to *in vivo* testing), then they are generally applied as an experimental treatment to test for probiotic effects. However, few studies have attempted to identify the underlying mechanisms involved in bacterial growth inhibition, or considered the wider competitive strategies employed and how this relates to the efficiency and mechanism of probiotic action *in vivo*. Although antagonism assays offer a limited representation of the diverse and complex interactions between probiotics and their associated host, microbial community and environmental factors, characterisation of these mechanisms is nonetheless necessary to ensure that probiotic use in shrimp aquaculture is both effective and sustainable. Furthermore, considering the rise of antimicrobial resistance (which is predicted to result in 10 million deaths by 2050 if the current trend continues; Meade *et al.*, 2020), the potential insights gained from such investigations extend far beyond the field of probiotics in shrimp aquaculture given that these probiotic species produce antimicrobial substances that have not yet been identified and which make them ideal candidates for bioprospecting for novel antimicrobial therapeutics. Here, after introducing the competitive exclusion principle and how it works through exploitation and interference competition, we provide a comprehensive overview of the mechanisms of competitive exclusion by species that have been screened and subsequently identified as probiotic based on their ability to inhibit the growth of pathogenic bacteria *in vitro*. We then go on to discuss how our current understanding of the mechanisms of competitive exclusion could be used to improve the selection and application of probiotics. Finally, we highlight important considerations and future directions for the selection and application of probiotics, with the aim to aid best practise in the development of effective microbial management strategies in shrimp aquaculture.

Competitive exclusion of pathogenic bacteria

Competitive exclusion is where co-occurring bacterial species in the same ecological niche compete for limited resources (nutrients and space) through two competitive strategies: exploitation and interference competition. Exploitation competition is indirect, characterised by rapid resource consumption (restricting supply to competitors and investing in growth), whereas interference competition occurs when one organism directly harms another, for example, through the production of antimicrobial compounds.

Exploitation competition includes mechanisms that increase, relative to the competitors, the rate at which nutrients are captured and utilised. Nutrient capture can be improved by the secretion of extracellular molecules that

break down complex macromolecules thus making nutrients more readily accessible. These extracellular excretion molecules include proteases (Bachmann *et al.*, 2011) and iron-chelating siderophores that access insoluble iron (Kümmerli *et al.*, 2015). These mechanisms can be considered co-operative traits, as the ‘public goods’ produced are accessible to all and may benefit a population (Morris *et al.*, 2012). Not all members of that population necessarily invest resources in the production of the compounds and the bacteria producing materials for enabling nutrient capture are vulnerable to inter- and intra-specific competition from social ‘cheaters’, who save resources and invest them in growth. For example, some bacteria can utilise heterologous siderophores, allowing them to save energy on production costs and reduce the accessibility of iron to the siderophore producing species (Khan *et al.*, 2006). The extent to which cheating occurs, however, appears to be dependent on environmental and social context (Ghoul *et al.*, 2014). Niehus *et al.* (2017) argue that although siderophores can be public goods (when all cells have the same receptors), they should be considered a competitive phenotype. Certainly, species able to produce ‘private’ siderophores with higher iron binding affinities have an advantage over lower-affinity siderophore producing organisms (Weaver & Kolter, 2004; Joshi *et al.*, 2006). The efficiency and speed with which microorganisms utilise nutrients can provide another competitive advantage. Optimal allocation of resources can maximise growth rates (Flamholz *et al.*, 2013; Bosdriesz *et al.*, 2015; Hui *et al.*, 2015). For example, switching between metabolic strategies depending on substrate availability, such as from the higher ATP-yielding respiration process to fermentation, even when glucose is available in excess, to maximise growth rates. This phenomenon, termed ‘overflow metabolism’ (Neijssel & Tempest, 1976; Molenaar *et al.*, 2009), has been observed in a number of fast-growing bacterial and fungal species including *Saccharomyces cerevisiae*, *Escherichia coli* and *Bacillus subtilis* (van Dijken *et al.*, 1993; Vemuri *et al.*, 2006; Sonenshein, 2007; Molenaar *et al.*, 2009; Basan *et al.*, 2015; LaCroix *et al.*, 2015). Whilst this mechanism may appear counter-intuitive, an increasing growth rate is the result of a trade-off between energy yield and synthesis rate of alternative pathways; fermentation requiring more carbon flux but being more proteome efficient (requires fewer proteins) than respiration, thereby allowing more resources to invest in growth (Molenaar *et al.*, 2009; Basan *et al.*, 2015).

Exploitation competition for space can be achieved through rapid colonisation of uninhabited niches, or through competing with populations that are already established. The production of adhesins and receptors that bind to specific surface features can provide a competitive advantage for the colonisation of unoccupied niches, as

well as prevent displacement by invaders (Schluter *et al.*, 2015). For example, after adherence to human epithelial cells, some *Lactobacillus* species produce extracellular glycoproteins, preventing pathogen attachment (Horie *et al.*, 2002; Golowczyc *et al.*, 2007; Johnson-henry *et al.*, 2007). Furthermore, attachment, whilst decreasing mobility, is critical for biofilm formation (surface-attached microbial communities; Hall-Stoodley *et al.*, 2004). The expansion of adhesive cells in biofilms can also aid the removal of non-adhesive cells from the population (Schluter *et al.*, 2015). Cell aggregation of the same genotype can also provide another competitive advantage by sharing resources, such as plasmids conferring antibiotic resistance (Savage *et al.*, 2013), limiting exposure to social ‘cheaters’ (West *et al.*, 2007; Smukalla *et al.*, 2008; Queller *et al.*, 2011; Drescher *et al.*, 2014), and increasing tolerance to antimicrobials (Olsen, 2015). However, there is strong competition within these biofilms (Xavier & Foster, 2007; Davies & Marques, 2009), in which nutrients become limited regionally (Stewart & Franklin, 2008; Kim *et al.*, 2014). On the other hand, to compete with biofilm formation, many species produce molecules that can actively stimulate dispersal of other species (without killing them), such as rhamnolipid and cis-2-decenoic acid (Irie *et al.*, 2005; Davies & Marques, 2009), within established biofilms. Alternatively, some species can disrupt biofilm formation. For example, *Pseudomonas aeruginosa* PsDAHP1 (isolated from the Indian prawn *Fenneropenaeus indicus*) has been found to antagonise the ability of *Vibrio parahaemolyticus* to form biofilms in zebrafish gills and intestine, resulting in a much looser architecture and possibly making them more susceptible to antimicrobials (Vinoj *et al.*, 2015).

Interference competition typically involves the production of antimicrobials, which range from strain-specific bacteriocins (ribosomally synthesised antimicrobial peptides; Yang *et al.*, 2014), to broad-spectrum antibiotics such as the quinolones (Aldred *et al.*, 2014). Investigation into the functional role of sub-inhibitory concentrations of antimicrobials has revealed their multifaceted nature (Fajardo & Martínez, 2008), with involvements in signalling (Davies *et al.*, 2006; Linares *et al.*, 2007; Romero *et al.*, 2011), inhibition of quorum sensing (Hong *et al.*, 2012; Algburi *et al.*, 2017) and kin recognition (Wall, 2016). Nevertheless, there is substantial experimental evidence to support the hypothesis that antibiotics are primarily used as weapons (Abrudan *et al.*, 2015; Cornforth & Foster, 2015). It is plausible that some species have evolved to use certain antimicrobials solely as weapons, whereas others have multiple functions. Interference competition also includes contact-dependent mechanisms such as Type V and Type VI secretion systems. The contact-dependent growth inhibition system (CDI) is a Type V system, first recognised in *Escherichia coli* (Aoki *et al.*, 2005), in which

polymorphic protein toxins (growth inhibitory signals) are delivered into the cytoplasm of the target cell displaying complementary receptors, mediating killing unless the recipient bacterium produces a corresponding antidote (Aoki *et al.*, 2005, 2010; Anderson *et al.*, 2012; Nikolakakis *et al.*, 2012). For example, the CdiA-CTEC869 tRNase toxin from enterohemorrhagic *E. coli* EC869 obstructs protein synthesis and thus inhibits growth by specifically cleaving transfer RNAs of recipient cells (Jones *et al.*, 2017). Recently, Garcia *et al.* (2016) have shown that, in *Burkholderia thailandensis*, gene expression and phenotypic changes that promote community-associated behaviours, such as biofilm formation, occur in immune (self) recipients in response to the CDI toxin. Their results suggest that the CDI system may have two functions, challenging heterospecific (non-immune) bacteria and promoting conspecific (immune) bacteria. Similarly, type VI secretion systems deliver effectors (substrates) directly into the cytoplasm of recipient cells (Cianfanelli *et al.*, 2016) resulting in death (Hood *et al.*, 2010; MacIntyre *et al.*, 2010; Schwarz *et al.*, 2010; Murdoch *et al.*, 2011; Russell *et al.*, 2014). Interestingly, the use of this system appears to be highly dynamic, with some species (such as *Vibrio cholerae*) employing highly offensive (non-directional) strategies (Basler *et al.*, 2012), while others, such as *P. aeruginosa*, orientate the machinery towards a perceived threat in a 'tit-for-tat' response (Basler *et al.*, 2013; Le Roux *et al.*, 2015). Furthermore, cell lysis via Type VI secretion systems may facilitate horizontal gene transfer, as DNA from the target cell may also be transferred into the attacking cell, potentially conferring benefits in the form of new genes, such as those conferring antimicrobial resistance, to the new host and providing a competitive advantage (Borgeaud *et al.*, 2015).

Probiotic bacteria

Considering the relative importance of bacterial antagonism assays for the screening of potential probiotics in shrimp aquaculture, investigations into the underlying mechanisms are limited. In the following sections, we discuss our current understanding of the mechanisms of pathogenic growth inhibition, *in vitro*, for individual strains within each genus of bacteria that has been identified as shrimp probiotic (Tables 1-3). We then go on to discuss the implications of the use of antagonism assays in probiotic screening, and how we can improve their efficiency and sustainability, *in vivo*, based on our current understanding of the mechanisms of competitive exclusion. We also highlight some of the key limitations and future directions for the methods used to identify probiotics in shrimp aquaculture.

Lactic acid bacteria (LAB)

Lactic acid bacteria produce many antibacterial substances including lactic acid, acetic acid, hydrogen peroxide and bacteriocins (Mokoena, 2017; Ringø *et al.*, 2020) that suppress growth of competing bacteria (Nes *et al.*, 2011; Reis *et al.*, 2012; Alvarez-Sieiro *et al.*, 2016). Many LAB have been identified as shrimp probiotics due to their ability to inhibit the growth of several pathogenic *Vibrio* species, *Aeromonas hydrophila* and *Pseudomonas fluorescens* through well and disc diffusion assays (Table 1), suggesting these species produce extracellular compounds with antimicrobial properties. The mechanisms of growth inhibition for LAB remain largely uncharacterised, but some studies have used catalase-treated and/or pH-neutralised cell-free culture supernatants (CFCS) to test for antagonistic activity and determine whether or not the source of growth inhibition was due to the effect of hydrogen peroxide and/or organic acid production, respectively (see Table 1). Kongnum and Hongpattarakere (2012) found that hydrogen peroxide was a major agent contributing to the antibacterial effect of *Lactobacillus plantarum* MRO3.12. Interestingly, Sgibnev and Kremleva (2017) found that hydrogen peroxide produced by probiotic vaginal lactobacilli were effective in increasing sensitivity to antibiotics, suggesting that hydrogen peroxide producing LAB in combination with antibiotic-producing species may be more effective for pathogenic growth inhibition. The antimicrobial activity of LAB shrimp probiotic *Streptococcus phocae* PI80 has been shown to be due to the production of the bacteriocin Phocaecin PI80, which inhibits the growth of a range of Gram-positive and Gram-negative bacteria, including *Listeria monocytogenes*, *Vibrio* spp., *Aeromonas hydrophila* and *Pseudomonas aeruginosa* (Satish Kumar & Arul, 2009). The authors of this work suggest that Phocaecin PI80 increases the permeability of sensitive cells through pore formation in the cytoplasmic membrane, as it was shown to induce potassium ion leakage in the tested indicator strains: *E. coli*, *Listeria monocytogenes* and *V. parahaemolyticus* (Satish Kumar & Arul, 2009).

Lactic acid bacteria have also been shown capable of inhibiting the adhesion of pathogenic bacteria to the intestinal mucosa of shrimp. For example, *Lactobacillus pentosus* HC-2 and *Enterococcus faecium* NRW-2 have been shown to adhere to crude intestinal shrimp mucus, suggesting that these strains may compete against pathogens such as *Vibrio* spp. for adherence sites in the intestinal mucus (Sha *et al.*, 2016c). The authors used fluorescent imaging to subsequently confirm that *L. pentosus* HC-2 was able to competitively exclude *V. parahaemolyticus* E1 in the intestine of the Whiteleg shrimp *Litopenaeus vannamei*. *L. pentosus* HC-2 was also shown to increase the level of transcription of the *luxS* gene in response to

V. parahaemolyticus E1, suggesting it may play an important role in improving its adherence to the gut of *L. vannamei* and in turn competitively exclude the adhesion of *V. parahaemolyticus* E1. These authors also reported that heat-killed *L. pentosus* HC-2 cells upregulated the expression of toxicity-related factors in *V. parahaemolyticus* E1 and suggested that shrimp fed a diet containing the intracellular components of *L. pentosus* HC-2 may result in an increase in the risk of infection by *Vibrio* spp. This hypothesis, however, was not then tested. Collectively, these findings illustrate the importance of understanding the underlying probiont-pathogen interactions, determining strain specific characteristics, and not extrapolating from related strains or even species. They also highlight that community analyses alone (for example through the use of 16S sequencing) are limited for assessments seeking to identify putative interactions.

Lactic acid bacteria are Generally Recognised as Safe (GRAS) by the USA Food and Drug Administration (US FDA), and currently have a wide range of applications in the human food chain, primarily in the manufacturing of fermented food products such as yogurt, cheese and alcoholic beverages. Similarly, the European Food Safety Agency (EFSA) includes many LAB species (belonging to the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus*) in the Qualified Presumption of Safety (QPS) list, meaning that, except for enterococci which no longer has QPS status, a demonstration of their safety only requires confirmation of the absence of determinants of resistance to antibiotics of human and veterinary clinical significance (EFSA BIOHAZ Panel, 2020). This would suggest that the use of LAB, with the exception of *Enterococcus faecium*, as probiotics is unlikely to have any significant potential health concern for human consumption of treated shrimp. There may, however, be safety concerns based on antibiotic resistance and virulence factors for multiple genus of LAB. This, in turn, highlights that a more thorough risk assessment may be required when considering the application of LAB as probiotics in shrimp aquaculture (Muñoz-Atienza *et al.*, 2013; Sharma *et al.*, 2016).

Bacillus

The genus *Bacillus*, like LAB, includes representatives that are defined as GRAS by the US FDA, but which, for the EFSA QPS qualification, are modified to include 'absence of food poisoning toxins, absence of surfactant activities, absence of enterotoxic activities' (EFSA BIOHAZ Panel, 2020). Therefore, *Bacillus* species would also seem to be ideal candidates for animals destined for human consumption, and indeed, they currently have a range of applications in food processing. *Bacillus* species also need to show an 'absence of acquired genes for antimicrobial resistance'

before introduction into the food chain (EFSA BIOHAZ Panel, 2020). The large majority of probiotics in shrimp aquaculture belong to the genus *Bacillus*, but their safety for use in shrimp aquaculture should not be presumed, and this should be the case with all isolates. For example, Guo *et al.* (2009) found that addition of *Bacillus cereus* biovar *toyoi* to cultures of the giant tiger prawn *Penaeus monodon* culture resulted in near total mass mortality. Similarly, *Bacillus subtilis* and *B. cereus* have been reported to cause bacterial white spot syndrome in cultured *P. monodon* (Wang *et al.*, 2000) and bacterial white patch disease in *L. vannamei*, respectively (Velmurugan *et al.*, 2015). This highlights the importance of thorough testing of probiotic candidates, and not simply extrapolating from related species or strains. Ngo *et al.* (2016) have shown that *Bacillus* strains isolated from shrimp intestines exhibit resistance to clinically relevant antibiotics, highlighting the importance of antibiotic susceptibility screening when evaluating shrimp probiotics. *Bacillus* produce a diverse array of over 20 different types of antimicrobial compounds (including polypeptide antibiotics, bacteriocins and lipopeptides), with a wide variety of activities ranging from antibacterial and antifungal, to anticancer and antiviral (Martirani *et al.*, 2002; Stein, 2005; Sutyak *et al.*, 2008; Smitha & Bhat, 2012; Mondol *et al.*, 2013; Cochrane & Vederas, 2016). Recently, genome mining of *Bacillus* spp. has successfully guided the identification and characterisation of novel antimicrobial metabolites (Yang *et al.*, 2016; Zhao & Kuipers, 2016), highlighting their competitive potential. Many studies investigating *Bacillus* species as potential probiotics in shrimp aquaculture (Soltani *et al.*, 2019) demonstrate antagonistic growth inhibition of shrimp, fish and human pathogens (Table 2). Most of these studies suggest that *Bacillus* species produce diffusible extracellular antimicrobials, through the inhibition of pathogens as assessed using the agar or well diffusion techniques and CFCS diffusion assays (Table 2). More specifically, the production of proteinaceous antibacterial substances (bacteriocins or bacteriocin-like inhibitory substances; BLIS, Zokaiefar *et al.*, 2012a,b; Masitoh *et al.*, 2016), antimicrobial polypeptides (AMPs; Cheng *et al.*, 2017; Cheng *et al.*, 2020) and antimicrobial lipopeptides (Lee *et al.*, 2016) have all been identified as a source of inhibition.

Recently, (Gao *et al.*, 2017) reported that *Bacillus pumilus* H2 produces an anti-*Vibrio* substance, structurally identical to amicoumacin A, that has been shown to inhibit the growth of 29 *Vibrio* strains by disrupting cell membranes and leading to cell lysis (Itoh *et al.*, 1981). No extracellular antimicrobial activity, however, was detected in *B. subtilis* BS11 and *Bacillus* sp. P11 when using an antimicrobial residue screening test kit (Powdchagun *et al.*, 2011; Utiswannahakul *et al.*, 2011) but this method tests only for '18 known standard antibiotics' (Utiswannahakul *et al.*, 2011). Tseng

et al. (2009) found that *B. subtilis* E20, isolated from the gut of *L. vannamei*, had no inhibitory effect against eight fish and shrimp pathogens and was only weakly inhibitory against *A. hydrophila* (Tseng *et al.*, 2009). Nevertheless, the authors found that a *B. subtilis* E20 supplemented diet increased resistance of *L. vannamei* to *Vibrio alginolyticus*. These results may be due to a variety of reasons, including an increased immune capability of the shrimp. Importantly, however, it helps illustrate that the disc diffusion method (using CFCS) alone may not capture the ability of *B. subtilis* E20 to inhibit growth using exploitation competition mechanisms. Illustrating this further, Luis-Villaseñor *et al.* (2015) showed that *Bacillus* strains, isolated from the intestinal tract of shrimp, can adhere to intestinal mucus and gastric mucin of *L. vannamei*, which may then confer an ability to exclude pathogens by competing for binding sites, as well as producing antimicrobial compounds. Similarly, *Bacillus licheniformis* DAHB1, isolated from *F. indicus*, has been shown to invest in exploitation mechanisms, producing a quorum-quenching N-hexanoyl-L-homoserine lactone (AHL)-lactonase (AiiA) able to inhibit vibrio biofilm development and colonisation of shrimp intestines (Vinoj *et al.*, 2014). More recent work has shown that *B. cereus* BP-MBRG/1b, isolated from the gut of a healthy giant freshwater prawn (*Macrobrachium rosenbergii*), is able to ablate both AHL signal molecules and quorum sensing in *A. hydrophila* (Wee *et al.*, 2018). Further effects relating to quorum sensing have been demonstrated. For example, *Bacillus* sp. NFMI-C has been shown to degrade N-hexanoyl-L-homoserine lactone (HHL) molecules (also produced by *A. hydrophila*) in co-culture, which subsequently increased the survival of *M. rosenbergii* larvae when challenged with *Vibrio campbellii*, whose virulence is regulated by AHL quorum sensing (Pande *et al.*, 2013, 2015). *Bacillus* spp. are known to produce intracellular lactonases, one of the two major classes of AHL-inactivating enzymes, that act by hydrolysing the lactone ring and are capable of inactivating a wide range of AHLs (Dong *et al.*, 2007; Pande *et al.*, 2015). The growth of *V. campbellii* was not affected by culture with *Bacillus* sp. NFMI-C or its CFCS, nor was the growth of the shrimp larvae affected when treated with *Bacillus* sp. NFMI-C. This would suggest that the probiotic effect was likely due to the ability of this isolate to degrade AHL through the production of lactase. This is of particular interest in the management of *Vibrio* spp. in shrimp aquaculture, whose ubiquitous and abundant presence in the shrimp gut (Holt *et al.*, 2020) plays an important role in the health of the animal, and not just in disease. *Vibrio* is the most dominant genus in the early development of *P. monodon* (Angthong *et al.*, 2020) and dominates in the gastrointestinal tract regardless of environment or life stage (Mongkol *et al.*, 2018). Probiotics that are theoretically able to prevent the onset of disease that have limited effects on

the microbial diversity of ecologically important genera warrant further investigation.

Vibrio

Cordero *et al.* (2012) showed that marine *Vibrio* populations are organised into socially cohesive units, in which interference competition is greater between (rather than within) ecologically defined populations. These findings suggest that the inhibitory activities of *Vibrio* species are strain-specific. The results from this study also indicate that low intra-population antagonism may result from fast allelic turnover and rapid loss and acquisition of genes. Therefore, considering virulence or antibiotic resistance of clinical importance, the use of *Vibrio* species in shrimp aquaculture may be unwise. Furthermore, these authors suggested that the antagonistic interactions investigated were due to small molecules and not proteins such as bacteriocins. Nevertheless, several vibriocins (bacteriocins produced by the genus *Vibrio*; Wahba, 1965; McCall & Sizemore, 1979; Sugita *et al.*, 1997; Shehane & Sizemore, 2002) and bacteriocin-like inhibitory substances (Prasad *et al.*, 2005; Carraturo *et al.*, 2006) involved in inter-strain and inter-species inhibition have been described. Several *Vibrio* species investigated for their probiotic potential in shrimp (Table 3) have been shown to produce diffusible extracellular antimicrobials, able to inhibit the growth of fish, shrimp and human pathogens (Gullian *et al.*, 2004; Balcázar *et al.*, 2007; Rahiman *et al.*, 2010; Pham *et al.*, 2014). These studies suggest that some *Vibrio* isolates may be good probiotics. The lack of QPS qualification, however, would suggest that there is currently not enough literature supporting the use of *Vibrio* species in animals intended for the food chain. Furthermore, it has been shown that the bacteriophage *Vibrio harveyi* myovirus like (Munro *et al.*, 2003) and *V. harveyi* siphophage 1 (Khemayan *et al.*, 2012) are able to confer and increase virulence of *V. harveyi* to shrimp, respectively. This is of concern when using uncharacterised *Vibrio* species in shrimp aquaculture (Table 3), as virulence may be easily acquired or transferred.

Pseudomonas

Pseudomonas spp. are known to produce a range of bioactive compounds, such as bacteriocins, pyocin, phenazinen, quinoline and quinolone (Isnansetyo & Kamei, 2009; Preetha *et al.*, 2010), and have been shown to antagonise the growth of several shrimp pathogens (Table 3). *Pseudomonas aeruginosa*, *Pseudomonas synxantha* (Van Hai *et al.*, 2007) and *Pseudomonas aestumarina* SLV22 (Balcázar *et al.*, 2007) produce diffusible extracellular antimicrobials that can inhibit the growth of several *Vibrio* pathogens; however, only few studies have characterised these

compounds. The anti-*Vibrio* activity of *P. aeruginosa* I-2, however, has been suggested to be attributed to a low molecular weight, non-proteinaceous antimicrobial compound, possibly pyocyanin (Chythanya *et al.*, 2002), that is a well-described virulence factor associated with disease in patients with cystic fibrosis (Jeffries *et al.*, 2016). If this is indeed the case, then it would raise safety concerns regarding use of this species in the food chain. *Pseudomonas sp.* W3, isolated from intensive shrimp pond water (Rattanachauy *et al.*, 2007), has been shown to produce proteolytic enzymes and lysozyme (N-acetylmuramidase; Rattanachauy *et al.*, 2010) and 2-heptyl-4-quinolone (HHQ) with anti-*Vibrio* activity (Rattanachauy *et al.*, 2011). Interestingly, *Pseudomonas sp.* W3 appears to have lost its ability to convert HHQ to PQS (2-heptyl-3-hydroxy-4(1H)-quinolone; Diggle *et al.*, 2006, 2007), a quorum sensing molecule involved in the production of iron-scavenging compounds (Diggle *et al.*, 2007; Rattanachauy *et al.*, 2011). Nevertheless, Reen *et al.* (2011) demonstrated strong antibacterial activity of HHQ against *Vibrio fischeri* and *Vibrio vulnificus*. The authors reported that this compound was bacteriostatic, in addition to reducing mobility and interfering with pellicle and biofilm activity in *B. subtilis*. This may raise concerns surrounding the efficiency of this species for use as probiotic treatment, particularly in combination with *Bacillus* species, as it is possible that it may affect *Bacillus sp.* that are already established in the gut. In contrast with this, high levels of siderophores from the catechol group were identified in cell-free extracts of *Pseudomonas* PS-102 (Vijayan *et al.*, 2006) able to inhibit the growth of several *Vibrio* and *Aeromonas* spp., suggesting investment in exploitation mechanisms. Similarly, CFCS of AHL-degrading *P. aeruginosa* strains, isolated from the intestine of *F. indicus*, was shown to inhibit more than 80% of biofilm formation by *V. parahaemolyticus* (Vinoj *et al.*, 2015). *Pseudomonas* spp. are known to produce acylases, the other major class of AHL-inactivating enzymes, which cleave the AHL molecule into homoserine lactone and a fatty acid (Fast & Tipton 2012), and exhibit substrate specificity (Tang & Zhang, 2014). Furthermore, the CFCS reduced the hydrophobicity index and exopolysaccharide production of *V. parahaemolyticus*, limiting biofilm formation and potentially increasing their susceptibility to antibiotics. *P. aeruginosa* is known to produce rhamnolipids and fatty acid messengers (such as *cis*-2-decenoic acid) that can disperse biofilms of several microbial species (Irie *et al.*, 2005; Davies & Marques, 2009). However, in this instance, the compound(s) was not characterised.

Pseudoalteromonas

Pseudoalteromonas spp. isolated from the marine intertidal areas of New Caledonia have shown growth inhibition

activity against *Vibrio nigripulchritudo* and *V. harveyi*, through the production of diffusible antimicrobials (Pham *et al.*, 2014). The antimicrobial compounds were not characterised in this study, but the authors noted that the isolates were coloured, and that pigmented species belonging to the *Pseudoalteromonas* genus are known to produce a variety of bioactive compounds (Bowman, 2007). Similarly, yellow colony-forming *Pseudoalteromonas* spp. CDM8 and CDA22 have been isolated from the hindgut of healthy *L. vannamei*, and shown to display antagonistic activity against pathogenic *V. parahaemolyticus* (Wang *et al.*, 2018). The antagonistic effect was abolished when the CFCS (for both species) was treated with catalase, suggesting that the inhibition was likely the result of hydrogen peroxide production. The two most predominant proteins isolated from the CFCS of CDM8 and CDA22 (identified by MALDI-TOF/TOF mass spectrometry), shared high similarity to a TonB-dependent receptor (TBDRs) and an antibacterial protein (L-lysine 6-oxidase) of *Pseudoalteromonas flavipulchra* JG1, respectively. TBDRs play a fundamental role in nutrient uptake, including iron-siderophore complexes (Moeck & Coulton, 1998), whilst the antimicrobial activity of L-lysine 6-oxidase has been attributed to its generation of hydrogen peroxide. In *Pseudoalteromonas tunicate*, the hydrogen peroxide generated by the lysine oxidase AlpP is involved in biofilm killing (Mai-Prochnow *et al.*, 2008). Interestingly, *Pseudoalteromonas sp.* CDM8 displayed weak antagonistic activity against *Pseudoalteromonas sp.* CDA22 and a noncooperative effect were observed for shrimp when fed with a combination of CDM8 and CDA22, highlighting the importance of investigating probiotic relationships in multi-strain/species treatments. Theoretically, similar species that occupy the same ecological niche within the shrimp gut will have likely developed strategies to compete with [competitively exclude] each other, suggesting that multi-strain probiotics would be better targeted to a wide range of niches.

Paenibacillus

Two isolates from the genus *Paenibacillus*, *P. polymyxa* (formerly recognised as *Bacillus polymyxa*) and an unassigned *Paenibacillus* spp., have shown probiotic effects in *L. vannamei* (Amoah *et al.*, 2020) and *P. monodon* (Ravi *et al.*, 2007), respectively. *P. polymyxa* are able to produce a variety of bioactive compounds, including lipopeptide biosurfactants able to disrupt biofilms (Quinn *et al.*, 2012). Even in their sporulated state, they can produce fusaricidins, AMPs and polymyxins due to the activity of non-ribosomal peptide synthetase systems (Shaheen *et al.*, 2011; Grady *et al.*, 2016). *Paenibacillus* spp. was shown to inhibit the growth of *Vibrio* species (including *V. harveyi*) when co-cultured using the well diffusion method, suggesting

that it is able to produce diffusible extracellular antimicrobials; however, the mechanism of antagonism was not investigated further.

Streptomyces

Species belonging to the genus *Streptomyces* produce a substantial number of bioactive compounds (over 7630; Bérdy, 2005) including antimicrobial, antifungal, anti-cancer agents (Bérdy, 2005; Kim *et al.*, 2008; Wang *et al.*, 2013; Zothanpuia *et al.*, 2017) and are good candidates for potential use as probiotics in aquaculture (Das *et al.*, 2010; Tan *et al.*, 2016). *Streptomyces* spp. N7 and RL8 isolated from marine sediment both have antagonistic activity against pathogenic *Vibrio* spp. (Bernal *et al.*, 2015). *Streptomyces* sp. N7 improved haemocyte counts, growth parameters and *Vibrio* counts in the shrimp hepatopancreas, but it also increased *Vibrio* counts in the rearing water and reduced the SOD activity. Furthermore, there was no difference in the survival rate of the treated shrimp when challenged with *V. parahaemolyticus*. Again, this highlights the importance of thorough testing of probiotic candidates, and not extrapolating from related species or strains. Interestingly, when *Streptomyces* spp. N7 and RL8 were combined, the negative effects on water *Vibrio* counts and survival rates were not counteracted by *Streptomyces* sp. RL8, albeit SOD activity returned to the level of that in the control group. This illustrates the importance of considering both the individual and the combined functional traits of probiotic candidates. Bernal *et al.* (2015) also showed that these strains are likely to adhere to the gut of their host, allowing them to competitively inhibit *Vibrio* spp. *in vitro*, in addition to the production of antimicrobials. Similarly, *Streptomyces* isolates and *Streptomyces rubrolavendula* biogranules (filamentous aggregation of cells) have demonstrated the ability to inhibit the growth of *Vibrio* spp. in co-culture (Das *et al.*, 2006, 2010; Augustine *et al.*, 2016); however, the mechanisms were not investigated.

Clostridium

Clostridium butyricum is the only member of the genus *Clostridium* tested as a probiotic in shrimp aquaculture (Table 3) and it shows several probiotic effects in shrimp, as it does in humans and other animals (Kanai *et al.*, 2015; Zhao *et al.*, 2017) including fish (Pan *et al.*, 2008; Gao *et al.*, 2013). The competitive exclusion mechanisms, however, have not been investigated (Table 3).

Psychrobacter

Few studies have been conducted on the use of *Psychrobacter* spp. as potential probiotics in aquaculture, and only

Psychrobacter sp. 17-1 has been reported as a probiotic for shrimp (Table 3). Screening of this isolate by the double layer method has suggested that it is able to produce extracellular antimicrobials, but the mechanism of antagonism against *V. harveyi* and *Aeromonas* sp. has not been investigated (Franco *et al.*, 2016).

Using competitive exclusion principles to enhance disease resistance

Despite the majority of probiotics in shrimp aquaculture being screened and selected for further testing based on their ability to antagonise bacterial shrimp pathogens *in vitro*, from the studies reported upon it is clear that relatively little is known about the mechanisms by which this growth inhibition occurs. Furthermore, the investment of energy into interference mechanisms (the production of extracellular antimicrobials) by shrimp probiotics suggests that they may be useful targets for bioprospecting for novel antimicrobial therapeutics. In the following sections, we discuss the implications and limitations of the use of antagonism assays to screen for potential probiotics.

Competitive exclusion principles and life strategies

Interference competition has been shown to improve species fitness (Rao *et al.*, 2005; Shank *et al.*, 2011) and stimulate biodiversity (Czárán *et al.*, 2002; Little *et al.*, 2008; Hibbing *et al.*, 2010). However, the energy investment involved in antimicrobial production generally results in slower growth rates and reduced rates of reproduction (Case & Gilpin, 1974; Little *et al.*, 2008). Closely related and co-occurring species can employ fundamentally different competitive strategies. To illustrate this, Patin *et al.* (2016) have shown that for two co-occurring marine Actinomycete species, *Salinispora arenicola* invests in interference competition via the production of antibiotics at the expense of growth, whereas *Salinispora tropica* invests in growth and exploitation competition. Copiotrophic species invest in rapid growth and are selected for in environments that are nutrient rich, whereas oligotrophic microbes are selected for in resource-poor environments (Koch, 2001; Roller & Schmidt, 2015). Roller and Schmidt (2015) defined a conceptual model proposing innate differences in growth efficiency, progeny per unit of resource utilised, between copiotrophic and oligotrophic microbial species and suggested that selection for efficiency based on resource availability and quality is a primary driver of microbial community composition. However, this model does not account for resources that are invested in interference competition mechanisms and may therefore result in population persistence regardless of the growth rate or efficiency. Furthermore, whilst this model shares many of the

underlying principles of r/K selection – the selection of combinations of traits in an organism that trade-off between quantity and quality of offspring – it does not consider environmental factors beyond resource availability. Thus, the use of functional traits to understand processes that influence community structure, such as the competitor/stress-tolerator/ruderal (CSR) theory (Grime & Pierce, 2012) are more applicable to developing microbial management strategies. The theory links community function and environmental conditions by classifying traits as Competitive (C; neighbours seek to capture the same unit of resource), Ruderal (R; assisting in re-establishment of population after disturbance) or Stress tolerant (S; tolerance of resource shortage, as opposed to resource competition) and proposes that prevailing traits will be a result of resource investment trade-off in such traits that confer an adaptive advantage to environmental disturbance and stress. Whilst the CSR theory was originally developed to explain plant communities, it has been successfully applied to soil microbial communities (Wood *et al.*, 2018). Using Grime's CSR theory, fast growing species investing in exploitation competition mechanisms (Ruderal life strategists) are selected for in disturbed environments that are not resource-limited, whereas slower growing species investing in interference mechanisms (competitive life strategists) are selected for in undisturbed environments where resources are not limiting (Wood *et al.*, 2018).

Considerations for the selection of shrimp probiotics

Most probiotics used in shrimp aquaculture appear to be competitive life strategists, as they are often selected for based on their ability to produce extracellular antimicrobials. Due to the nature of shrimp aquaculture, in which ponds are disturbed by chemical treatments, weather events and disease, these probiotics may not always be the most effective choice. Rather, certain environmental conditions and disturbances are likely to favour the proliferation of ruderal life strategists. To compensate for this, frequent treatment may be necessary to establish the probiotic (competitive life strategist) within the microbial community at the required abundance and achieve the desired effects. This may in turn impact the microbial community structure of the system and further favour ruderal life strategists that invest in exploitation mechanisms. Improper use of probiotic treatments may potentially lead to conditions which favour pathogenic invasion, for example by using species that compete with indigenous beneficial species, or alter the overall structure or function of the community (Long & Azam, 2001; Pérez-Gutiérrez *et al.*, 2013). Therefore, elucidating the mechanisms underlying growth efficiency and competitive strategies employed by potentially

probiotic and pathogenic species, as well as understanding their optimal environmental conditions, are needed to develop the most effective microbial management strategies to both prevent and treat disease. For example, tailoring treatments so that they contain species, or strains, that can competitively exclude specific pathogens using multiple mechanisms will increase the likelihood of successful pathogen exclusion; particularly if the pathogen becomes resistant to a mechanism, or there is a new (or opportunistic) pathogenic invasion. Patin *et al.* (2016) designed a workflow that aims to determine whether interference or exploitation competition is employed by using a series of simple inhibition assays. Following a direct challenge (co-culture) assay, a cell-free agar (and disc) diffusion assay determines if the antagonistic activity can be attributed to a diffusible molecule and thus is indicative of antimicrobial production. A negative result suggests the production of non-diffusible growth inhibitors, indicating an exploitation mechanism is at work. In this case, the assay is followed with a supplement assay in which the direct challenge assay is repeated with media supplemented by specific nutrients, such as iron. Applying this workflow provides a simple and cost-effective way to assess the competitive strategy likely employed by probiotic species *in vivo*, although future studies should consider the extent to which *in vitro* models (mechanisms of antagonism) are accurate representations of their interactions with pathogens, and other competitors, *in vivo*. Making this distinction is of importance when considering the type (i.e. desired characteristics) of probiotic to apply to specific conditions; for example, to improve disease resistance before a pathogenic invasion, or in response to an environmental disturbance, such as disease. Theoretically, competitive life strategists would be more suited to prophylactic treatment, as they are selected for in undisturbed environments, whilst ruderal life strategists would be best applied to reduce the severity of disease, as they are selected for in disturbed environments. This also highlights the importance of applying ecological theory to develop effective probiotics for disease prevention in shrimp aquaculture.

Potential probiotic identification based on the use of limited methods, such as cell-free extracts or diffusion assays exclusively, is selective of interference-based competition strategies due to the antimicrobial (and diffusible) properties of the extracellular metabolites. The culture conditions of potential probiotics may not stimulate inhibitory compound production (Long & Azam, 2001), or increase the activity of the compound (Rattanachua *et al.*, 2011). Furthermore, the inhibitory effect of cell-free supernatants and resistance of pathogens may be incorrectly determined due to insufficient concentrations in assays such as the disc-diffusion method (Smith, 2006; Van Hai *et al.*, 2007). The limitations of culture-based probiotic selection

methods, combined with the current focus on the ability of probiotic species to inhibit the growth of *Vibrio* species (Table 3), may overlook the capability of many putative probiotic species for shrimp aquaculture. Whilst pathogenic *Vibrio* species are undoubtedly an important target for potential probiotics to antagonise, screening based on *in vitro* growth inhibition of a limited number of specific pathogens (i.e. *Vibrio*) may be inadvertently polarising the type (i.e. competitive strategy) of shrimp probiotics available. Therefore, closer attention to characterising the underlying mechanisms by which probiotics inhibit the growth of these pathogens *in vitro*, combined with our increasing understanding of their effects *in vivo*, will enable us to better evaluate and develop our screening and selection methods. Probiotic treatment is able to alter the microbial community structure in the shrimp gut and confer resistance to pathogens such as *V. parahaemolyticus*, as is the case for *Bacillus aryabhattai* TBRC8450 treatment in *L. vannamei* (Tepaamorndech *et al.*, 2019). Probiotics targeted specifically at *Vibrio* spp. may increase the risk of disease in the event of an invasion by another species that the probiotic cannot inhibit, doing so by lowering the probability that other members of that community provide antagonistic traits. Stimulation of the shrimp immune response by such probiotics should, theoretically, mitigate some of these effects. However, it remains to be tested that a probiotic with such effects is able to enhance resistance to multiple pathogens. Rather, the current trend is to identify novel probiotics, which, without focusing on the underlying mechanisms, will continue to create a 'never-ending story' in this field (Ringø *et al.*, 2016). We suggest, therefore, that emphasis should be placed on the development of complementary, culture-independent, methods of potential probiotic identification and characterisation. With the increasing affordability of sequencing approaches, whole genome sequencing of probiotic species would benefit our understanding of the potential mechanisms of probiotic effects and allow us to make more informed decisions about the suitability of such species. Furthermore, metagenomic, transcriptomic, and metatranscriptomic studies, complemented by proteomics, would allow for more thorough investigation, assessment and development of probiotic treatments in shrimp aquaculture.

Conclusions and future perspectives

Probiotics in shrimp aquaculture are often screened and selected for *in vivo* testing based on their ability to competitively exclude pathogens *in vitro*. Few studies have attempted to identify the underlying mechanisms involved in bacterial growth inhibition; however, they collectively illustrate the importance of understanding the underlying probiotic-pathogen interactions, determining strain specific

characteristics and not extrapolating from related strains or even species. This is particularly important to consider when assessing the safety and suitability of a probiotic species for use in animals intended for human consumption, and consideration should be given to investigating the potential impact of probiotic treatment on the virulence of pathogens. The application of complementary sequencing approaches such as whole-genome sequencing of probiotic species will aid this assessment. The antagonism assays employed to screen for probiotics in shrimp aquaculture use a limited number of pathogens (i.e. *Vibrio*) and preferentially select for interference-based mechanisms, which may overlook the probiotic potential of many species and possibly increase the risk of pathogen invasion. To address this, future work should focus on expanding probiotic screening methods (including the development of culture-independent approaches) to include more species that use exploitation-based mechanisms and pathogens with distinct mechanisms of pathogenesis. Tailoring treatments so that they contain species, or strains, that can competitively exclude specific pathogens using multiple mechanisms will increase the likelihood of successful pathogen exclusion; particularly if the pathogen becomes resistant to a mechanism, or there is a new (or opportunistic) pathogenic invasion. The impact of probiotic treatment on the shrimp gut microbiota and disease resistance should also be carefully considered as not to reduce the abundance of other ecologically important species. Special consideration should be given to identifying and testing probiotics that are able to prevent the onset of disease with limited effects on the microbial community structure and function. Furthermore, the efficiency of treatment may be improved by considering the competitive life strategy of the probiotic species. Future studies should investigate this by validating the competitive strategies used *in vivo*, and designing pathogen challenge trials that compare the effects of probiotic administration, prior to and at the onset of disease or other such environmental disturbances, on the shrimp gut microbiome and disease resistance.

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