Interaction of *Aeromonas* Strains with Lactic Acid Bacteria via Caco-2 Cells

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The genus *Aeromonas* includes some species that have now been identified as human pathogens of significant medical importance. We investigated the ability of 13 selected *Aeromonas* strains belonging to nine species isolated from clinical cases ($n = 5$), environmental waters ($n = 5$), and fish ($n = 3$) to adhere to and translocate Caco-2 cells in the absence and presence of two lactic acid bacteria (LAB), i.e., *Lactobacillus acidophilus* and *Bifidobacterium breve*. *Aeromonas* isolates were also assessed for their cytotoxicity, the presence of virulence genes, and hemolysin production. Among the clinical isolates, one strain of *Aeromonas veronii* biovar *veronii* and two strains of *Aeromonas hydrophila* carried cytotoxin (*act*), heat-labile toxin (*alt*), hemolysin (*hlyA*), and aerolysin (*aerA*) genes, were cytotoxic to Vero cells, produced hemolysin, and showed higher adherence to Caco-2 cells. In contrast, this was seen in only one environmental strain, a strain of *A. veronii* biovar *sobria*. When *Aeromonas* strains were coinoculated with LAB onto Caco-2 cells, their level of adhesion was reduced. However, their rate of translocation in the presence of LAB increased and was significantly ($P < 0.05$) higher among fish strains. We suggest that either the interaction between *Aeromonas* and LAB strains could have a detrimental effect on the Caco-2 cells, allowing the *Aeromonas* to translocate more readily, or the presence of the LAB stimulated the *Aeromonas* strains to produce more toxins and/or increase their translocation rate.

Aeromonas species are ubiquitous in aquatic environments and are also isolated from food products, including dairy, fish, and vegetables (1–3). There are currently 28 recognized species (4); however, *Aeromonas* taxonomy is a controversial issue, with the status of a few species still requiring clarification (5). Nonetheless, some *Aeromonas* species have long been recognized as fish pathogens, with a few species, mainly *Aeromonas hydrophila*, *A. caviae*, *A. schubertii*, and *A. veronii* biovar *sobria*, now recognized as human pathogens. Most often, these cause wound and urinary tract infections, gastroenteritis, and septicemia (5).

Virulence of *Aeromonas* can be attributed to a few factors, including adhesion to epithelial cells, mediated through flagella or outer membrane protein structures (6–8), host cytoskeletal rearrangement (9), and translocation in a cell culture model (10). *Aeromonas* strains are also capable of producing toxins. Generally, these are either hemolytic or enterotoxigenic and include aerolysin (*aerA*), hemolysin (*hlyA*) (11), *Aeromonas* heat-labile enterotoxin (*Alt*), heat stable toxin (*Ast*), and cytotoxin (*Act*) (12).

Probiotics, such as *Lactobacillus* and *Bifidobacterium* species, have been shown to provide protection against pathogens by competing for attachment sites (13) and through the production of bacteriocins. It has been suggested that *Aeromonas* strains from different sources may be virulent regardless of their origin (14, 15). The interaction of probiotics with *Aeromonas* species has predominantly focused on aquatic animals (16), and very little is known about their interaction with human cells.

In view of the above, this study was undertaken to assess the ability of different *Aeromonas* species from clinical and environmental sources as well as fish to interact with a human gut epithelium cell model and the ability of lactic acid bacteria (LAB) to competitively inhibit their adhesion and translocation.

MATERIALS AND METHODS

***Aeromonas* strains.** Clinical isolates of *Aeromonas* included three type culture strains (*A. schubertii* ATCC 43700, *A. jandaei* ATCC 49568, and *A. veronii* biovar *veronii* ATCC 35624) and two isolates obtained from a local

metropolitan hospital (*A. hydrophila* RBH1 and *A. hydrophila* RBH2). The environmental strains were part of a bigger collection of *Aeromonas* strains isolated from a local river system, of which we included four strains previously shown to belong to different common groups and that were adherent to Caco-2 cells (10). These include *A. veronii* biovar *sobria* 5Ag, *A. veronii* biovar *sobria* 7Bg, *A. hydrophila* 10Bb, and *A. caviae* 15Df. The type culture collection strain *A. media* ATCC 33907, isolated from fish farm effluent, was also included in this study. The fish isolates included three culture collection strains: *A. schubertii* 298 92/0140, *A. eucrenophila* ATCC 23309, and *A. bestiarum* CRW50.

Lactic acid bacteria. Lactic acid bacteria (LAB) included five known strains of *Lactobacillus* and two strains of *Bifidobacterium*, all obtained from Elizabeth Norin (Karolinska Institute, Stockholm, Sweden), and included *Lactobacillus acidophilus*, *L. delbrueckii*, *L. paracasei*, *L. plantarum* 299V, *L. reuteri*, *Bifidobacterium bifidum*, and *B. breve*. All strains were maintained at -80°C in tryptic soy broth (TSB; Oxoid Australia) or de Man, Rogosa, Sharpe (MRS; Oxoid Australia) broth supplemented with 20% glycerol. Working cultures were maintained on tryptic soy agar (TSA) plates or MRS plates grown at 37°C for 24 h.

Hemolysin production. Hemolysin production was determined visually after streaking *Aeromonas* strains on 5% horse blood agar plates (17), with Columbia agar base (Oxoid, Australia), following overnight incubation at 37°C .

DNA extractions. Total DNA from *Aeromonas* isolates was extracted using a Qiagen DNeasy blood and tissue extraction kit (Qiagen, Australia) following the manufacturer's instructions, with the final elution in 200 μl of the provided buffer AE. DNA quality from the isolates was visually assessed using gel electrophoresis using a 1% agarose gel in $0.6\times$ Tris-borate-EDTA (TBE) buffer that was prestained with ethidium bromide

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TABLE 1 Primer sequences and PCR conditions used in this study

Primer	Sequence (5'–3') ^a	Primer concn (μM)	Template DNA (μl)	Expected product size (bp)	Reference
<i>aerA</i>	F: GCCTGAGCGAGAAGGT R: CAGTCCCACCCACTTC	0.2	1	416	17
<i>hlyA</i>	F: GGCCGGTGGCCGAAGATACGGG R: GGCGGCGCCGACGAGACGGG	0.2	1	597	17
<i>act^b</i>	F: GAGAAGGTGACCACCAAGAACA R: AACTGACATCGGCCCTTGAAGTC	0.2	2.5	232	12
<i>alt^b</i>	F: TGCTGGGCTGCGTCTGGCGGT R: AGGAAGCTGTTGACGAAGCAGG	0.2	2.5	361	12
<i>ast^b</i>	F: GACTTCAATCGCTTCTCAACG R: GCATCGAAGTCACTGGTGAAGC	0.2	2.5	536	12

^a F, forward; R, reverse.^b Run in multiplex.

and run at 130 V for 60 min. Bands were visualized under UV. Extracted DNA was then stored at –20°C until tested.

Detection of virulence genes. All PCRs were performed in an Eppendorf master gradient thermal cycler (Eppendorf, Australia). All PCRs included 1× MangoMix (Bioline, Australia), primers, and template (Table 1), using double-distilled water (ddH₂O) to reach a final reaction volume of 25 μl. The thermocycling protocol for *aerA* and *hlyA* was performed as previously described (11). A multiplex PCR was run for the *alt*, *ast*, and *act* genes as previously described (12) but with a shortened initial denaturation step of 95°C for 5 min. PCR products were visualized under UV on a 2% agarose gel, prestained with ethidium bromide, in 0.6× TBE buffer run at 130 V for ca. 60 min.

Bacteriocin production. LAB strains were assessed for their ability to produce bacteriocin-like substances to effectively inhibit the growth of the *Aeromonas* strains. A modification of the agar overlay method by Kiliç et al. (18) was used to allow rapid screening of all isolates against the *Aeromonas* strains. LAB were grown in MRS broth overnight at 37°C, and ca. 1 × 10⁸ CFU of each strain was spread onto MRS agar plates and incubated for 24 h at 37°C. Agar blocks (0.5 cm by 0.5 cm) were aseptically cut and overlaid on TSA plates, which had been seeded with 1 × 10⁸ CFU of each *Aeromonas* strain and incubated at 37°C for 24 h. Bacteriocin production was evaluated by measuring the resulting zone of inhibition. Isolates were considered positive for bacteriocin production if a clear inhibition zone larger than 2 mm from the edge of the agar block was observed (18).

Cytotoxicity assay. Vero cells (ATCC CCL-81), derived from African green monkey kidney cells, were grown to confluence in Eagle's minimal essential medium (EMEM) (Lonza, Australia) substituted with 10% fetal bovine serum (FBS; Lonza, Australia) in a cell culture-treated 96-well plate (Nunc, Australia). Bacterial suspensions from an overnight growth in 5 ml of double-strength TSB were prepared as previously described by Fiorentini et al. (19), with the exception that cell-free supernatant was obtained with a 0.22-μm syringe filter. Cell-free supernatant was diluted to 1:10 and 1:100 in sterile phosphate-buffered saline (PBS), pH 7.4, and 100 μl was inoculated into each well containing confluent Vero cells in 200 μl of antibiotic-free growth medium. Vero cells were observed for a cytopathic effect (CPE) at 4, 24, and 48 h after inoculation using an inverted phase-contrast microscope (magnification, ×400). Cell cytotoxicity was determined when a CPE was observed in >50% of Vero cells at a dilution of 1:10 or greater (19). All tests were performed in triplicate.

Adhesion assay. The ability of LAB strains to adhere to Caco-2 cells was initially tested, and two strains, one each of *L. acidophilus* and *B. breve*, were selected based on their high levels of adherence and used in competitive assays against *Aeromonas* isolates.

Caco-2 cells (ATCC HTB-37), human colon adenocarcinoma cells,

were grown on glass coverslips (12 mm in diameter, 1 mm thick) to 75% confluence in Roswell Park Memorial Institute (RPMI) 1640 medium (Lonza, Australia) supplemented with 10% FBS and 1% penicillin-streptomycin (Lonza, Australia) in a 24-well culture plate (Nunc, Australia) at 37°C in 5% CO₂. Before the adhesion assay, cells were rinsed three times with 1 ml of RPMI 1640 to remove residual antibiotics and replaced with antibiotic-free culture medium. LAB isolates were grown in MRS broth, whereas *Aeromonas* strains were grown in TSB, both with agitation (110 rpm) for 4 h at 37°C, after which bacterial suspensions were centrifuged at 3,220 × g for 12 min. The bacterial pellets were resuspended in sterile PBS, and Caco-2 cells were infected with 10⁸ CFU of bacteria per well and incubated at 37°C in 5% CO₂ for 1.5 h. Nonadherent bacteria were removed by washing cells three times with sterile PBS. Cells were then fixed with 95% ethanol for 5 min, air dried, Gram stained, and examined by light microscopy (magnification, ×1,000). Adhesion was determined based on previous methods (20). Strains that showed adhesion to less than 10% of the cells (as was seen in the negative control, *Escherichia coli* strain JM109) were regarded as nonadherent (20). All tests were performed in duplicate.

Competitive adhesion assay. Two different approaches were used to assess the impact of LAB on adhesion of *Aeromonas* strains. In the first method, i.e., competitive adhesion assay, each of the LAB strains (10⁸ CFU per well) was individually inoculated concurrently with the *Aeromonas* strains as described by Collado et al. (21). In the second method, i.e., the exclusion adhesion assay, the LAB strains (10⁸ CFU per well) were inoculated onto Caco-2 cells 1.5 h prior to inoculation of the *Aeromonas* strains (10⁸ CFU per well). In both methods, bacteria and cells were incubated and stained as described above.

Translocation assay. A two-compartment model was used to assess the ability of *Aeromonas* strains to translocate. Briefly, Caco-2 cells were grown in well inserts (Millipore, Australia) with a porous membrane, 8 μm, in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin (Lonza, Australia) to confluence (21 days). Confluence was determined by assessing stability of the transepithelial electrical resistance (TEER). Prior to bacterial inoculation, the medium in the outer well was replaced with 800 μl of medium without antibiotics, while the medium in the inner well was replaced with 200 μl of RPMI without antibiotics. One hundred microliters of bacterial suspension (i.e., 10⁸ CFU per well) was then inoculated into each insert, the TEER value was measured, and a 100-μl sample of the medium in the outer well was collected (time zero) before incubation at 37°C. At time intervals of 30, 60, 90, and 120 min, samples were collected from the outer well, and the inserts were transferred into new wells each containing 800 μl of fresh medium. The number of translocating bacteria was determined using the spread plate technique. Competitive translocation was carried out by in-

TABLE 2 Adhesion to Caco-2 cells and bacteriocin production of seven LAB strains against 13 *Aeromonas* clinical, environmental, and fish isolates

		Bacteriocin activity against <i>Aeromonas</i> strain ^a												
Lactic acid bacterium	No. of adhering bacteria per cell	Clinical					Environmental					Fish		
		534	567	568	RBH1	RBH2	5Ag	7Bg	10Bb	15Df	565	298	563	899
<i>L. acidophilus</i>	7 ± 5	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>L. delbrueckii</i>	7 ± 4	—	—	+	—	+	+	—	—	—	—	+	—	—
<i>L. paracasei</i>	2 ± 1	+	—	+	—	+	+	—	—	—	—	+	+	—
<i>L. plantarum</i> 299V	4 ± 2	—	—	+	—	+	+	—	—	—	—	+	+	—
<i>L. reuteri</i>	4 ± 3	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>B. bifidum</i>	7 ± 6	—	—	+	—	+	+	—	—	—	—	+	+	—
<i>B. breve</i>	7 ± 4	+	—	+	—	+	+	—	—	—	—	+	—	—

^a Strains of *L. acidophilus* and *B. breve* were chosen for competitive adhesion and translocation. 534 and 298, *A. schubertii*; 567, *A. jandaei*; 568, *A. veronii* biovar veronii; RBH 1, RBH 2, and 10Bb, *A. hydrophila*; 5Ag and 7Bg, *A. veronii* biovar sobria; 15Df, *A. caviae*; 565, *A. media*; 563, *A. eucrenophila*; and 899, *A. bestiarum*.

oculating 10⁸ CFU per well of each of the LAB and the *Aeromonas* strain concurrently. All translocation assays were performed in duplicate (10).

Statistical analysis. A nonparametric one-way analysis of variance (ANOVA), the Kruskal-Wallis test, was performed to determine the statistical difference between the mean (± standard deviation [SD]) number of adhering bacteria, and a two-way ANOVA with Tukey's multiple comparison was used for translocating bacteria using the GraphPad Prism 6 (version 6.02 for Windows) software.

RESULTS

Selection of LAB strains for competitive adhesion assays. Two strains, one each of *L. acidophilus* and *B. breve*, showed higher adherence to Caco-2 cells than other LAB strains (Table 2) and were therefore used for competitive adhesion and translocation studies. *L. acidophilus* displayed no bacteriocin production against any *Aeromonas* strains, unlike *B. breve*, which showed activity against at least one *Aeromonas* strain from each source (Table 2).

None of the LAB tested showed cytotoxicity toward Vero cells as part of the selection criteria for translocation studies.

Virulence properties of *Aeromonas* strains. Three clinical strains of *Aeromonas*, i.e., two strains of *A. hydrophila* and one strain of *A. veronii* biovar veronii as well as one environmental strain of *A. veronii* biovar sobria, showed at least five of the seven virulence properties tested and were cytotoxic to Vero cells (Table 3). These strains also showed beta-hemolysis on blood agar, while the hemolysin gene (*hlyA*) was found in only two strains (Table 3).

In general, clinical strains of *Aeromonas* were significantly more adherent (12 ± 6.6 CFU/cell) than environmental strains (5 ± 1.1 CFU/cell) or fish strains (9 ± 6.2 CFU/cell) ($P < 0.0001$), although this was not consistent among the individual strains. For instance, the strains with the highest overall levels of adherence were two *A. schubertii* strains, one each from a clinical case and fish flora (Table 3). Adhesion could not be determined for two strains,

TABLE 3 Adhesion of *Aeromonas* strains to Caco-2 cells in the absence and presence (competitive and exclusion adhesion) of selected lactic acid bacteria

Type and species (strain)	No. of adherent cells (CFU/cell)	% change in:										Cytotoxin production	Beta-hemolysis on blood agar
		Competitive adhesion		Exclusion adhesion		Virulence genes							
		<i>B. breve</i>		<i>B. breve</i>	<i>L. acidophilus</i>	<i>act</i>	<i>alt</i>	<i>ast</i>	<i>hlyA</i>	<i>aerA</i>			
Clinical strains	12 ± 6.6												
<i>A. schubertii</i> (534)	23 ± 12.6	−77	−86	−84	−	+	−	−	−	−	+		
<i>A. jandaei</i> (567)	6 ± 4.3	+8	−79	−22	−	−	−	−	−	−	−		
<i>A. veronii</i> biovar veronii (568)	11 ± 5.5	−61	−46	−15	+	+	−	−	+	+	+		
<i>A. hydrophila</i> (RBH 1)	8 ± 4.6	−57	Lysed	−28	+	+	−	+	+	+	+		
<i>A. hydrophila</i> (RBH 2)	Lysed	Lysed	Lysed	Lysed	+	+	−	−	+	+	+		
Environmental strains	5 ± 1.1												
<i>A. veronii</i> biovar sobria (5Ag)	Lysed	Lysed	Lysed	Lysed	−	+	−	+	+	+	+		
<i>A. veronii</i> biovar sobria (7Bg)	7 ± 3.1	−26	−16	+84	−	−	−	−	−	−	−		
<i>A. hydrophila</i> (10Bb)	5 ± 4.7	−33	−22	+42	−	−	−	−	−	−	−		
<i>A. caviae</i> (15Df)	5 ± 3.4	−15	−41	−19	−	−	−	−	−	−	−		
<i>A. media</i> (565)	4 ± 2.1	+14	+3	−14	−	−	−	−	−	−	−		
Fish strains	9 ± 6.2												
<i>A. schubertii</i> (298)	17 ± 9.1	−64	−84	−75	−	−	−	−	−	−	−		
<i>A. eucrenophila</i> (563)	7 ± 7.5	−53	−53	−19	−	−	−	−	+	−	−		
<i>A. bestiarum</i> (899)	2 ± 1.5	+132	+53	+83	−	−	−	+	−	−	−		

Virulence genes tested for included those encoding *Aeromonas* cytotoxin (*act*), *Aeromonas* heat-labile toxin (*alt*), *Aeromonas* heat-stable toxin (*ast*), hemolysin (*hlyA*), and aerolysin (*aerA*).

TABLE 4 Rate of translocation of *Aeromonas* strains through Caco-2 cells^a

Type and species (strain)	No. of translocating bacteria (10 ³ CFU/ml) at time (min)				Total
	30	60	90	120	
Clinical					
<i>A. schubertii</i> 534	425	714	117	262	1,518
<i>A. jandaei</i> 567	23	120	178	75	396
<i>A. veronii</i> biovar veronii 568	7	8	46	28	89
Environmental					
<i>A. veronii</i> biovar sobria 7Bg	88	29	39	171	327
<i>A. hydrophila</i> 10Bb	56	19	9	85	169
<i>A. caviae</i> 15Df	2	15	10	0	27
<i>A. media</i> 565	65	99	677	53	894
Fish					
<i>A. schubertii</i> 298	4	96	8	28	136
<i>A. eucrenophila</i> 563	210	383	278	342	1,213
<i>A. bestiarum</i> 899	403	890	1,340	278	2,911

^a *Aeromonas* strains (5Ag, RBH1, and RBH2) that lysed cells during adhesion assays are not included.

A. hydrophila RBH 2 and *A. veronii* biovar sobria 5Ag, as these strains lysed Caco-2 cells in both adhesion and translocation studies. A diffuse pattern of adhesion to Caco-2 cells was observed in all strains.

In both the competitive adhesion (concurrent inoculation of

the LAB and *Aeromonas* strains) and exclusion (inoculation of LAB prior to *Aeromonas* strains) assays, there was a decrease in the number of bacteria adhering to Caco-2 cells, although this was not common to all strains (Table 3). Furthermore, when *L. acidophilus* was inoculated concurrently with *Aeromonas* strains, Caco-2 cells lysed (Table 3).

Competitive translocation assay. There was a difference in the ability of *Aeromonas* strains to translocate through Caco-2 cells, and this was irrespective of their sources. In general, *Aeromonas* strains isolated from fish translocated more than clinical or environmental strains (Table 4). Concurrent inoculation of LAB and *Aeromonas* strains also indicated that the degree of translocation of the clinical and the environmental strains was not affected with the presence of either LAB strain (Fig. 1). However, there was a marked increase in translocation of *Aeromonas* from fish in the presence of LAB strains from the early stages of the assay. Throughout the experiment, the integrity of the cell layer remained intact, with the TEER only slightly decreasing (Fig. 1).

DISCUSSION

Regardless of their origin, *Aeromonas* strains may possess virulence factors (3, 14, 15). In this study, three clinical and one environmental strain of *Aeromonas* were shown to carry four out of five virulence genes tested. These strains were also cytotoxic to Vero cells and produced hemolysin. The cytotoxicity of these

FIG 1 Transepithelial electrical resistance (TEER) and number of translocating *Aeromonas* bacteria among strains of clinical, environmental, and fish origin in the presence and absence of *Bifidobacterium breve* and *Lactobacillus acidophilus*.

strains, at least in two cases, caused total lysis of Caco-2 cells, and therefore, it was not possible to assess their level of adhesion to Caco-2 cells. LAB have long been used to confer health benefits to humans as a food additive and possibly as a prophylactic treatment (22). These bacteria have been shown to be able to competitively exclude intestinal pathogens by adhering to gut epithelium and producing bacteriocins (13). At present, little is known of the effects of these peptides against *Aeromonas* species. It has been shown that bacteriocin produced by *L. lactis* was capable of inhibiting growth of *Aeromonas* strains *in vitro* (23). The authors of that study proposed a potential application of this LAB in the food industry whereby vegetables can be treated with bacteriocins to control *Aeromonas* populations. In our study, we also found that strains of *L. delbrueckii*, *L. paracasei*, *L. plantarum* 299V, *B. bifidum*, and *B. breve* displayed bacteriocin activity against selected *Aeromonas* strains. While the production of bacteriocin was shown to be *Aeromonas* strain specific, it was seen that bacteriocin inhibition occurred regardless of the source and species of *Aeromonas*, suggesting their potential in food industry, as also suggested by others (23).

A number of toxin genes have been found in *Aeromonas* strains (10). Using a multiplex PCR to detect three enterotoxin genes (*act*, *alt*, and *ast*) of *Aeromonas*, Bin Kingombe and colleagues (12) showed that the *act* and *alt* genes were the most prevalent genes in different species of *Aeromonas* isolated from foods. In our study, we also found that apart from two clinical strains, one environmental strain of *A. veronii* biovar *sobria* also carried these genes. These strains were also found to carry *aerA* and *hlyA*, which are also reported to be produced by virulent strains of *A. hydrophila* (11). We also found a fish isolate, *A. bestiarum*, carrying the *hlyA* gene, although this gene was not phenotypically expressed on agar plates.

Adhesion to the gastrointestinal (GI) tract is thought to be an important step toward initiating infection (8). The Caco-2 cell line used in this study has been shown to be a suitable model to assess the interaction of *Aeromonas* with GI epithelium of humans (24). Our results were in agreement with previously reported findings that *Aeromonas* strains readily adhere to the Caco-2 cell monolayer (25). Those workers, however, tested the adhesion of only two species of *Aeromonas*. In our study, we found that all tested species of *Aeromonas* could adhere to this cell line, although the level of adhesion differed among the species. Of these, *A. eucrenophila* and *A. bestiarum* are considered environmental species with little or no association with human or fish disease (5). While our data suggest the ability of these strains to colonize human gut epithelium, this does not necessarily indicate their potential to cause intestinal diseases, as none of the virulence genes (except *hlyA*) tested were found in these strains.

Adhesion to enterocytes is thought to be the precursor to bacterial translocation (26, 27). All *Aeromonas* strains in this study showed the ability to translocate in the two-compartment Caco-2 cell culture. However, strains of fish origin showed an overall higher level of translocation than clinical or environmental isolates. Jutfelt et al. (28) showed that the fish pathogen *A. salmonicida* could readily translocate through intestinal segments of rainbow trout *in vitro* (28). These workers suggested that a membrane-bound surface factor, such as the type III secretion system, may be involved, as they demonstrated lower translocation rates in heat-inactivated *A. salmonicida* (28). With the lack of any phenotypically expressed toxin genes among our fish isolates, we suggest a

similar mode of translocation. This however, needs to be confirmed.

When the adhesion of *Aeromonas* strains to Caco-2 cells was tested in the presence of LAB, there was a reduction in the adherence of these strains. A similar result was found by Balcázar et al. (29), who tested the ability of three LAB strains to competitively inhibit adhesion of a range of fish pathogens to skin and intestinal mucus of rainbow trout (29). Despite this, in our study, the overall translocation rate of *Aeromonas* strains increased in the presence of both LAB strains, a result that was unexpected. The reason for this is not clear to us. We postulate that this was not due to a better ability of *Aeromonas* to adhere to Caco-2 cells, as there was a reduction in adhesion of these strains to the Caco-2 cells in the adhesion assay. We also rule out an increase in the cell monolayer permeability, as the TEER values remained relatively stable throughout the assay. However, it is possible that this effect could be partly due to the higher bacterial concentration relative to surface area between the adhesion assay and the translocation assay, in which the well inserts had a smaller surface area. Based on these results, we suggest that *Aeromonas* strains, regardless of their origin or virulence properties, can translocate in the human gut epithelial cell model, such as the Caco-2 cell line, and this ability is increased in the presence of some LAB strains. Furthermore, some of the virulence properties found in clinical isolates/species can also be found in environmental or fish strains. We also suggest that assessment of the ability of LAB strains to act as probiotics should not be based on the results of competitive adhesion assay alone and that their ability to competitively inhibit translocation of these bacteria should be also evaluated before any conclusion can be made.

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REFERENCES

- Gonzalez-Rodriguez MN, Sanz JJ, Santos JA, Otero A, Garcia-Lopez ML. 2002. Foodborne pathogenic bacteria in prepackaged fresh retail portions of farmed rainbow trout and salmon stored at 3°C. *Int. J. Food Microbiol.* 76: 135–141. [http://dx.doi.org/10.1016/S0168-1605\(02\)00011-9](http://dx.doi.org/10.1016/S0168-1605(02)00011-9).
- Martins LM, Marquez RF, Yano T. 2002. Incidence of toxic *Aeromonas* isolated from food and human infection. *FEMS Immunol. Med. Microbiol.* 32:237–242. <http://dx.doi.org/10.1111/j.1574-695X.2002.tb00559.x>.
- McMahon MAS, Wilson IG. 2001. The occurrence of enteric pathogens and *Aeromonas* species in organic vegetables. *Int. J. Food Microbiol.* 70: 155–162. [http://dx.doi.org/10.1016/S0168-1605\(01\)00535-9](http://dx.doi.org/10.1016/S0168-1605(01)00535-9).
- Euzéby JP. 1997. List of bacterial names with standing in nomenclature: a folder available on the internet. *Int. J. Syst. Bacteriol.* 47:590–592. (List of Prokaryotic Names with Standing in Nomenclature [<http://www.bacterio.net>]). <http://dx.doi.org/10.1099/00207713-47-2-590>.
- Janda JM, Abbott SL. 2010. The genus *Aeromonas*: taxonomy, pathogenicity, and infection. *Clin. Microbiol. Rev.* 23:35–73. <http://dx.doi.org/10.1128/CMR.00039-09>.
- Canals R, Ramirez S, Vilches S, Horsburgh G, Shaw JG, Tomas JM, Merino S. 2006. Polar flagellum biogenesis in *Aeromonas hydrophila*. *J. Bacteriol.* 188:542–555. <http://dx.doi.org/10.1128/JB.188.2.542-555.2006>.
- Kirov SM, O'Donovan LA, Sanderson K. 1999. Functional characterization of type IV pili expressed on diarrhea-associated isolates of *Aeromonas* species. *Infect. Immun.* 67:5447–5454.
- Vazquez-Juarez RC, Romero MJ, Ascencio F. 2004. Adhesive properties of a LamB-like outer-membrane protein and its contribution to *Aeromonas veronii* adhesion. *J. Appl. Microbiol.* 96:700–708. <http://dx.doi.org/10.1111/j.1365-2672.2004.02177.x>.
- Low KW, Goh SG, Lim TM, Sin YM, Leung KY. 1998. Actin rearrangements accompanying *Aeromonas hydrophila* entry into cultured fish cells. *J. Fish Dis.* 21:55–65. <http://dx.doi.org/10.1046/j.1365-2761.1998.00076.x>.

10. Snowden L, Wernbacher L, Stenzel D, Tucker J, McKay D, O'Brien M, Katouli M. 2006. Prevalence of environmental *Aeromonas* in South East Queensland, Australia: a study of their interactions with human monolayer Caco-2 cells. *J. Appl. Microbiol.* 101:964–975. <http://dx.doi.org/10.1111/j.1365-2672.2006.02919.x>.
11. Heuzenroeder MW, Wong CYF, Flower RLP. 1999. Distribution of two hemolytic toxin genes in clinical and environmental isolates of *Aeromonas* spp.: correlation with virulence in a suckling mouse model. *FEMS Microbiol. Lett.* 174:131–136. <http://dx.doi.org/10.1111/j.1574-6968.1999.tb13559.x>.
12. Bin Kingombe CI, D'Aoust JY, Huys G, Hofmann L, Rao M, Kwan J. 2010. Multiplex PCR method for detection of three *Aeromonas* enterotoxin genes. *Appl. Environ. Microbiol.* 76:425–433. <http://dx.doi.org/10.1128/AEM.01357-09>.
13. Collado MC, Meriluoto J, Salminen S. 2007. In vitro analysis of probiotic strain combinations to inhibit pathogen adhesion to human intestinal mucus. *Food Res. Int.* 40:629–636. <http://dx.doi.org/10.1016/j.foodres.2006.11.007>.
14. Khajanchi BK, Fadl AA, Borchardt MA, Berg RL, Horneman AJ, Stemper ME, Joseph SW, Moyer NP, Sha J, Chopra AK. 2010. Distribution of virulence factors and molecular fingerprinting of *Aeromonas* species isolates from water and clinical samples: suggestive evidence of water-to-human transmission. *Appl. Environ. Microbiol.* 76:2313–2325. <http://dx.doi.org/10.1128/AEM.02535-09>.
15. Ottaviani D, Parlani C, Cittero B, Masini L, Leoni F, Canonici C, Sabatini L, Bruscolini F, Pianetti A. 2011. Putative virulence properties of *Aeromonas* strains isolated from food, environmental and clinical sources in Italy: a comparative study. *Int. J. Food Microbiol.* 144:538–545. <http://dx.doi.org/10.1016/j.ijfoodmicro.2010.11.020>.
16. Gudmundsdóttir BK, Björnsdóttir B. 2007. Vaccination against atypical furunculosis and winter ulcer disease of fish. *Vaccine* 25:5512–5523. <http://dx.doi.org/10.1016/j.vaccine.2007.02.009>.
17. Wong CYF, Heuzenroeder MW, Flower RLP. 1998. Inactivation of two haemolytic toxin genes in *Aeromonas hydrophila* attenuates virulence in a suckling mouse model. *Microbiology* 144:291–298. <http://dx.doi.org/10.1099/00221287-144-2-291>.
18. Kiliç AO, Pavlova SI, Ma WG, Tao L. 1996. Analysis of *Lactobacillus* phages and bacteriocins in American dairy products and characterization of a phage isolated from yogurt. *Appl. Environ. Microbiol.* 62:2111–2116.
19. Fiorentini C, Barbieri E, Falzano L, Matarrese P, Baffone W, Pianetti A, Katouli M, Kuhn Möllby IR, Bruscolini F, Casiere A, Donelli G. 1998. Occurrence, diversity and pathogenicity of mesophilic *Aeromonas* in estuarine waters of the Italian coast of the Adriatic Sea. *J. Appl. Microbiol.* 85:501–511. <http://dx.doi.org/10.1046/j.1365-2672.1998.853517.x>.
20. Grey PA, Kirov SM. 1993. Adherence to HEp-2 cells and enteropathogenic potential of *Aeromonas* spp. *Epidemiol. Infect.* 110:279–287. <http://dx.doi.org/10.1017/S0950268800068217>.
21. Collado MC, Gueimonde M, Hernandez M, Sanz Y, Salminen S. 2005. Adhesion of selected *Bifidobacterium* strains to human intestinal mucus and the role of adhesion in enteropathogen exclusion. *J. Food. Prot.* 68:2672–2678.
22. O'Flaherty S, Klaenhammer TR. 2010. The role and potential of probiotic bacteria in the gut, and the communication between gut microflora and gut/host. *Int. Dairy J.* 20:262–268. <http://dx.doi.org/10.1016/j.idairyj.2009.11.011>.
23. Kumar M, Jain AK, Ghosh M, Ganguli A. 2012. Potential application of an anti-*Aeromonas* bacteriocin *Lactococcus lactis* ssp. *lactis* in the preservation of vegetable salad. *J. Food Saf.* 32:369–378. <http://dx.doi.org/10.1111/j.1745-4565.2012.00389.x>.
24. Kirov S, Hayward L, Nerrie M. 1995. Adhesion of *Aeromonas* sp. to cell lines used as models for intestinal adhesion. *Epidemiol. Infect.* 115:465–473. <http://dx.doi.org/10.1017/S0950268800058623>.
25. Couto CRA, Oliveira SS, Querioz MLP, Freitas-Almeida AC. 2007. Interactions of clinical and environmental *Aeromonas* isolates with Caco-2 and HT29 intestinal epithelial cells. *Lett. Appl. Microbiol.* 45:405–410. <http://dx.doi.org/10.1111/j.1472-765X.2007.02199.x>.
26. Mangell P, Lennernaes P, Wang M, Olsson C, Ahrne S, Molin G, Thorlacius H, Jeppsson B. 2006. Adhesive capability of *Lactobacillus plantarum* 299V is important for preventing bacterial translocation in endotoxemic rats. *APMIS* 114:611–618. http://dx.doi.org/10.1111/j.1600-0463.2006.apm_369.x.
27. Ringø E, Myklebust R, Mayhew TM, Olsen RE. 2007. Bacterial translocation and pathogenesis in the digestive tract of larvae and fry. *Aquaculture* 268:251–264. <http://dx.doi.org/10.1016/j.aquaculture.2007.04.047>.
28. Jutfelt F, Sundh H, Glette J, Mellander L, Throndur Björnsson B, Sundell K. 2008. The involvement of *Aeromonas salmonicida* virulence factors in bacterial translocation across the rainbow trout, *Oncorhynchus mykiss* (Walbaum), intestine. *J. Fish Dis.* 31:141–151. <http://dx.doi.org/10.1111/j.1365-2761.2007.00879.x>.
29. Balcázar JL, Vendrell D, de Blas I, Ruiz-Zarzuela I, Gironés O, Múzquiz JL. 2007. *In vitro* competitive adhesion and production of antagonistic compounds by lactic acid bacteria against fish pathogens. *Vet. Microbiol.* 122:373–380. <http://dx.doi.org/10.1016/j.vetmic.2007.01.023>.