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Studies into the potential application of probiotic bacteria as feed supplements for commercially cultured Crustacea, primarily the Pacific white shrimp, *Litopenaeus vannamei*

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Abstract

A significant problem faced by the aquaculture industry is the loss of stock through infection and disease. Cultured animals, particularly larval and post-larval stages, suffer increased incidences of disease primarily as a result of the high population densities at which they are stocked. Thus far the typical approach to bacterial pathogen control in the majority of commercial crustacean aquaculture facilities involves the prophylactic (and often incorrect) use of antimicrobials, i.e. antibiotics and chemotherapeutic agents. These are however costly to develop, limited in their application and most significantly are instrumental in creating antibiotic resistant strains.

Many current theories in crustacean pathogen control embrace a multifaceted approach, often combining the therapeutic use of antibiotics/chemotherapeutic agents with the administration of probiotic bacteria and immunostimulants and improved farm management. The purpose of this project was to identify any benefits and potential mechanisms by which probiotic bacteria may act on the health/growth parameters of the commercial aquaculture species, *Litopenaeus vannamei* and European shore crab, *Carcinus maenas*. Work was also undertaken to isolate and identify bacteria from healthy shrimp microbiota that may be of use as probiotics, with the possibility of commercial application within the industry. *In vitro* methods were utilised for screening potential probiotics for inhibitory activity against crustacean pathogens, followed by a series of *in vivo* trials to assess the effects of probiotic feed enrichment on the gut bacterial population and growth parameters. Molecular techniques were utilised to elucidate any effects probiotic administration may have on the bacterial community structure of the gut of *L. vannamei*.

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Contents.

Acknowledgements

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Chapter 1

General Introduction

1.1 Taxonomy and geographical distribution of species investigated

Carcinus maenas and *Litopenaeus vannamei* are members of subphylum Crustacea of the phylum Arthropoda. The subphylum Crustacea contains 38,000 known species and is the only major group of aquatic arthropods; it includes the crabs, shrimps, prawns lobsters and crayfish. The aforementioned crustaceans are all members of order Decapoda, which comprises *ca.* 25% of recorded crustacean species.

Carcinus maenas, the European green shore crab, is a member of infraorder Brachyura (the true crabs) of suborder Pleocyemata. It is a native European species, however, it is also an invasive species on the east and west coasts of the United States, South America, Australia and South Africa (Cohen et al, 1995; Kuris & Lafferty, 1996; Roman & Palumbi, 2004; Ahyong, 2005). It is an intertidal species capable of surviving in all types of protected and semi-protected marine and estuarine habitats (NIMPIS, 2002).

The family Penaeidae of suborder Dendrobranchiata, is a family of prawns (often referred to as the penaeid shrimp) containing many species of economic importance. Currently, the most significant of these species is the Pacific white shrimp (also known as the Whiteleg shrimp), *Litopenaeus vannamei.* Pacific white shrimp are native to the eastern Pacific region, with a range extending from Mexico to Peru (Valles-Jimenez et al, 2004). *L. vannamei* are also extensively cultured in Southeast Asia, Indonesia and South & Central America (http://fao.org/2009).

1.2 Species biology – *Carcinus maenas*

C. maenas is a littoral/sublittoral species found living on numerous intertidal substrate types ranging from dense, submerged aquatic vegetation to fine mud (Cohen & Carlton, 1995). Typically soft sediments are preferred as the animal relies extensively on camouflage to avoid predation and often buries itself. Studies of the cytochrome *c* oxidase I (COI) gene expressed genetic differentiation between European populations indicating that adult *C. maenas* is unable to cross areas of deeper water (Roman & Palumbi, 2004). Therefore, as with the majority of decapod species it relies principally on a pelagic larval stage for species distribution (Grosholz, 1996). There is the facility for the distribution of adult animals via anthropogenic means, e.g. via ballast water, on ships hulls and in packing materials (i.e. seaweeds). The appearance of *C. maenas* as an invasive species in Australia, South Africa and the Americas correlates with such human maritime activity. The species is physiologically hardy, being both euryhaline, tolerating salinities from 4 to 52 ppt, and eurythermic, surviving in temperatures from 0 to 30°C (Cohen and Carlton 1995). The species is adaptable, capable of surviving air exposure for 10 days, and this, coupled with a high fecundity and the ability to exploit numerous food sources (being an omnivorous scavenger) makes *C. maenas* a highly successful marine organism (Roman & Palumbi, 2004). Despite the existence of a small European fishery (*ca.* 1200 tonnes annually, predominantly in the UK and France) *C. maenas* is not usually regarded as a commercially important species (http://fao.org/2009).

1.2.1 The external anatomy of *C. maenas* (see Figure 1)

C. maenas possesses five pairs of thoracic appendages modified as walking legs or pereopods, anterior to which are an additional three pairs modified as maxillipeds. This arrangement is a primary morphological characteristic of decapod crustaceans (Ruppert & Barnes, 1994). Of the five walking legs the first pair are enlarged and chelated and are referred to as chelipeds. *C. maenas* possess a well developed finely granular carapace up to 60 mm long and 80 mm wide and bearing five acuminate antero-lateral teeth (Figure 1). The frontal region, between the eyestalks, bears three additional rounded, rostrum teeth. The chelipeds may also bear small black spots arranged longitudinally. Carapace colouration may vary from dark reddish brown to a matt grey green, sometimes with yellow granules present (NIMPIS, 2002). This colour variation has a genetic component but is largely due to local environmental factors (Brian et al, 2005). The reduced and flattened abdominal region is folded beneath the thorax (a characteristic of brachyuran crabs) and is typically a pale yellow/green in males and a dark brown/green in females. This abdominal colouration coupled with the female's smaller size and wider abdomen can be used to distinguish the sexes.

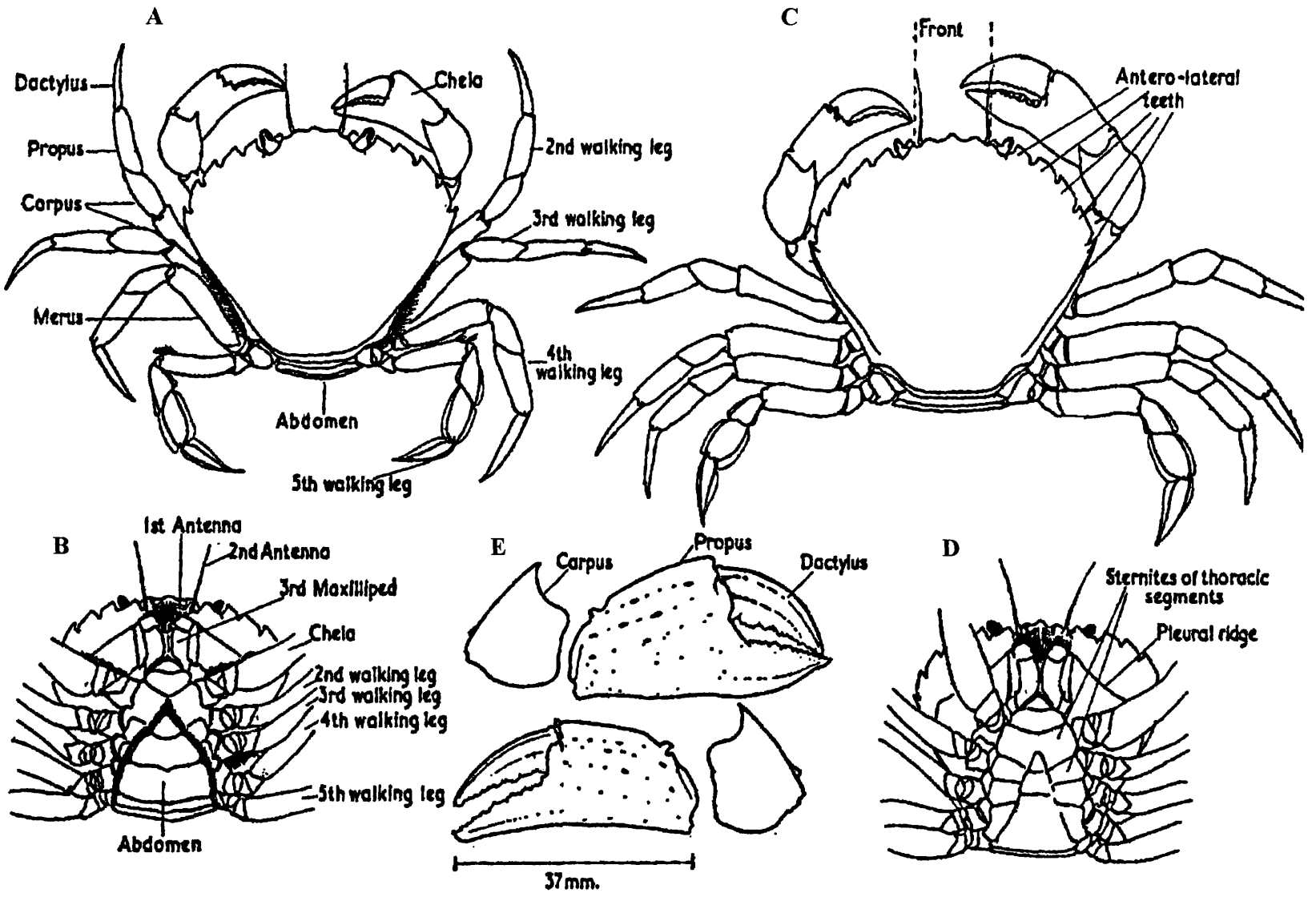


Figure 1. External features of *Carcinus maenas* (adapted from Crothers, 1976).
(**A**) Female, dorsal view. (**B**) Female, ventral view. (**C**) Male, dorsal view. (**D**) Male, ventral view. (**E**) Chelae of adult male.

1.2.2 The internal anatomy of *C. maenas* (see Figures 2, 3 & 4)

The alimentary canal in brachyurans (as for the majority of crustaceans) is short, straight and relatively simple. It can be divided into three general regions; the fore, mid and hind guts (Ruppert & Barnes, 1994). The fore gut is involved in rending and milling ingested material and acts as a triturating stomach. The anteroventral mouth opens into a short oesophagus which in turn expands into a large anterior section or cardiac stomach containing an array of opposing chitinous ridges, denticles and calcareous ossicles. The function of this chamber is the maceration and homogenisation of ingested material (Ruppert & Barnes, 1994). The mid gut is responsible for the majority of digestion and absorption and comprises the posterior region of the stomach (also referred to as the pyloric stomach) which opens into the hepatopancreas via two large ducts. The hepatopancreas is a large, spongy, bi-lobed digestive gland composed of ducts and blind ending secretory tubules. The hepatopancreas performs a similar function to the vertebrate pancreas and ileum. As well as being the primary source of digestive enzymes, it also functions as a primary storage organ, containing cells responsible for glycogen, lipid and calcium storage (Stanier et al, 1968; Ruppert & Barnes, 1994). In *C. maenas* the two lobes of the hepatopancreas extend outwards from the digestive tract along the upper anterior wall of the carapace. The tubule walls of the hepatopancreas are a single-cell epithelium composed of four distinct cell types (Figure 2); Embryonic cells (E-cells), Fibrillar cells (F-cells), Blister or extrusion cells (B-cells) and Resorptive cells (R-cells) (Stanier et al, 1968; Corrêa Jr et al, 2002). These cell types occur in different lateral regions of the tubules; i.e. the further from the distal tip the greater the cell differentiation (Al-Mohanna & Nott, 1989). E-cells are located at the distal ends of

the organ's tubules and are small and undifferentiated; they are the only cells in the hepatopancreas where mitoses are observed. The E-cells are generally regarded as precursor cells, giving rise to the Fibrillar cells (Köhler et al, 1998). The mid-region of the tubule contains F, R & B cells (Stanier et al, 1968) (Figure 2). F-cells contain large amounts of rough endoplasmic reticulum and numerous vacuoles and subsequently take up material for intracellular digestion and differentiate into B-cells (Al-Mohanna & Nott, 1989). B-cells are characterised by a single large vacuole (i.e. is an F-cell where smaller vacuoles have coalesced) and pinocytose material from the lumen for intracellular digestion (Figure 2) (Al-Mohanna & Nott, 1989). At the end of digestion the B-cells are then extruded into the lumen, aging B-cells are eliminated from the epithelium and incorporated into the faeces (Al-Mohanna & Nott, 1989; Köhler et al, 1998). The R-cells are responsible for absorbing soluble nutrients from the tubule lumen and storing reserve substances such as glycogen and lipid (Al-Mohanna & Nott, 1989; Köhler et al, 1998; Sousa et al, 2005). The proximal region of the tubule contains some aging B-cells, but is dominated by R-cells (Al-Mohanna & Nott, 1989). Different regions of the R-cells are active at different phases of the digestive cycle, early in the cycle it is believed that they take up material (i.e. glycogen, lipid and ions of copper, zinc, phosphorus and sulphur) from the lumen via diffusion (Al-Mohanna & Nott, 1989). In addition R-cells take up surplus material from the haemolymph, via pinocytosis and diffusion, as shown by Al-Mohanna & Nott (1987).

A dorsal chamber posterior to the pyloric stomach, known as the dorsal caecum, is also believed to produce digestive secretions. From the mid gut the ingested material moves into the hindgut where any remaining useful products of digestion are absorbed and the waste material is compacted into faeces. At this point a second chamber

branches off the hindgut, the hindgut caecum (Figure 4B), however, the function of this chamber remains unclear (McLaughlin, 1980). For detail on the microbiota of the G.I. tract of decapod crustaceans see Section 1.4.

The open circulatory system of *C. maenas* is extensive; the box-shaped heart is dorsal and anchored to the dorsal body wall/exoskeleton. Haemolymph enters the heart via three openings or ostia and exits via seven arteries. These arteries undergo numerous branching to supply the animal's various organs and tissues. The circulatory system in *C. maenas* is an open system. After the haemolymph enters the tissue sinuses it eventually drains into a ventral sternal sinus from which it is returned to the gills for oxygenation. From the gills the haemolymph is returned to the pericardium and heart via branchiopericardial vessels (Ruppert & Barnes, 1994).

C. maenas possess nine pairs of phyllobranchiate gills housed laterally in two brachial chambers; each gill is composed of a central axis along which lateral extensions are arranged. Each axis possesses an afferent and efferent branchial channel, haemolymph flows to each lamella via the afferent channel and exits via the efferent. The forward positioned inhalant opening in brachyuran crabs means the water takes a U-shaped course thorough the gill chambers. The flow of water over the lamellae is maintained by the beating of scapognathites or 'gill bailers'. The haemolymph of *C. maenas* contains cells in the form of haemocytes as well as various dissolved substances such as gases, nutrients, metabolites, hormones, waste products and the copper-based respiratory pigment haemocyanin (Bachère et al, 2004). Three morphologically distinct types of haemocyte are present in the haemolymph of decapod crustaceans (Figure 3); granulocytes, semi-granulocytes and agranular hyaline cells (Bauchau, 1981). Typically, *C. maenas* haemolymph possesses a total cell concentration of 2-4 x 10^7 cells ml^{-1} (Powell & Rowley, 2007). Variation in circulating haemocyte

populations can be due to numerous factors, both environmental and physiological. The three haemocyte types vary in their functions; the granular cells (i.e. granulocytes) are regarded as being involved with prophenoloxidase activity and cytotoxicity; hyaline cells are responsible for phagocytosis, while semi-granular type cells (semi-granulocytes) are regarded as an intermediate stage between these two cell types, with a corresponding overlap in function (Söderhäll & Smith, 1983; Johansson et al, 2000; Vogan & Rowley, 2002; Smith et al, 2003). The majority of circulating haemocytes are semi-granular (*ca.* 80%), with the granular (*ca.* 15%) and hyaline (*ca.* 5%) comprising the remainder (Powell & Rowley, 2007).

Both the testes and ovaries are similarly positioned in *C. maenas* (as is the case in most brachyuran crabs) and lie dorsally in the cephalothorax. The gonads are paired and extend outwards from the midline along the dorsal surface of the hepatopancreas.

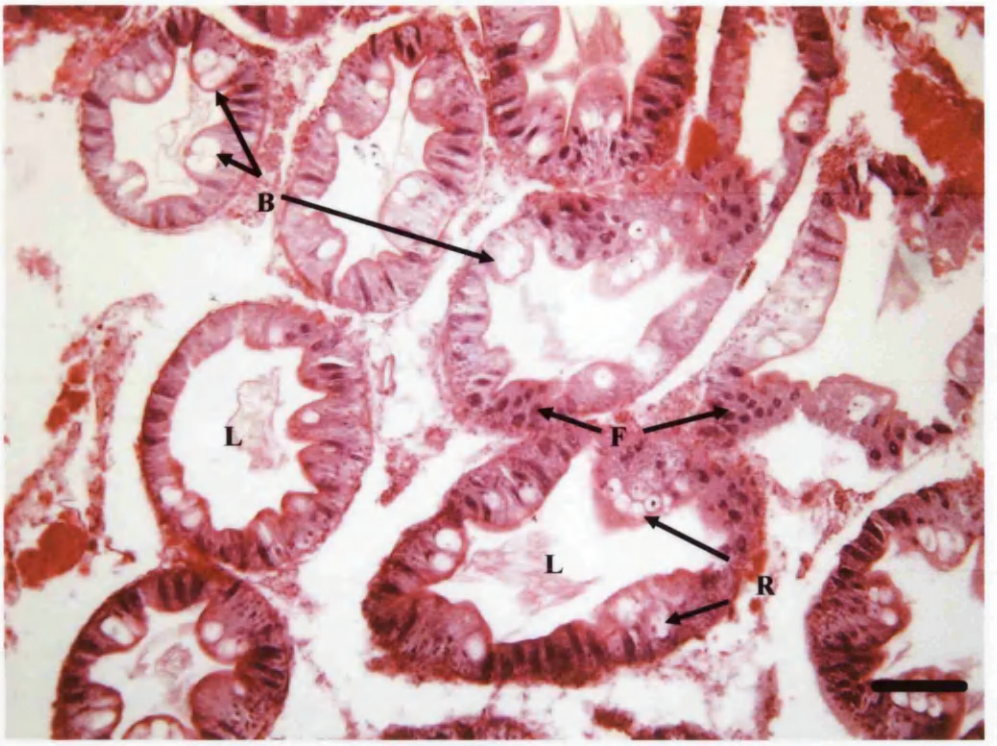


Figure 2. Mid power photomicrograph displaying tubules of the hepatopancreas of the shore crab *Carcinus maenas* in cross section; The arrows indicate the three cell types making up the tubule (excluding the embryonic (E) cells located at the distal tips of each tubule), B-cells (**B**), F-cells (**F**) and R-cells (**R**). (**L**) indicates the tubule lumen. (Scale bar = 100 µm). Material was fixed in Bouin's and stained with haematoxylin and eosin. Micrograph courtesy of F. Eddy.

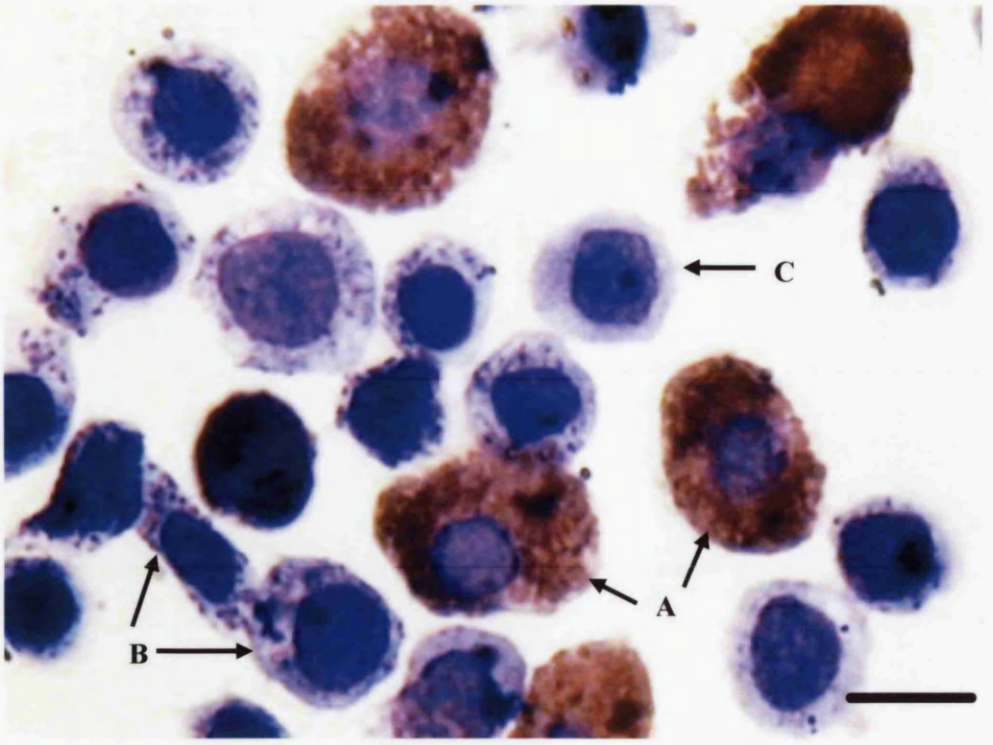


Figure 3. Wright's stained haemocytes of *Carcinus maenas*. Photomicrograph displaying the three main haemocyte cell types; indicated by arrows, (**A**) granulocytes, (**B**) semi-granulocytes and (**C**) agranular hyaline cells. (Scale bar = 10 µm). Micrograph courtesy of F. Eddy.

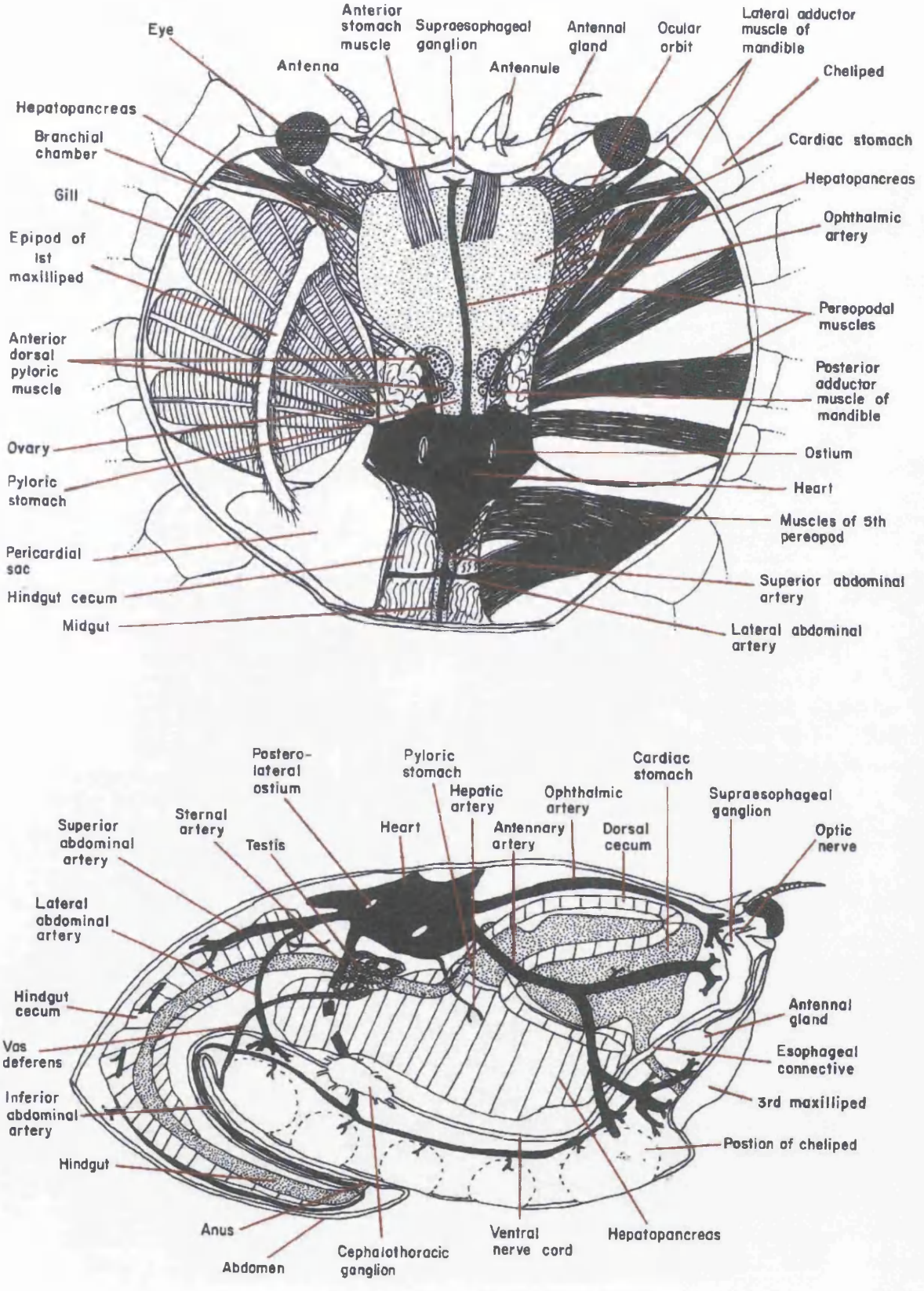


Figure 4. Internal anatomy of a typical brachyuran crab (from McLaughlin, 1980). (**A**) Dorsal view of animal with upper carapace removed. (**B**) Lateral view of animal bisected along midline; position of the chelipeds indicated by broken lines.

1.2.3 Reproduction and life cycle of *C. maenas* (refer to Figure 5)

In *C. maenas*, as in all decapods, sperm is transmitted from male to female via spermatophores. Transfer is performed via ejaculatory ducts located at the base coxa of the last leg of the male. The male attends to the female premoult and carries her beneath him (ventral surface to ventral surface), releasing her during moult (which occurs typically in summer, although this may vary with local conditions) and copulating shortly there after. The males typically moult post-copulation (Ruppert & Barnes, 1994). During copulation the spermatophores are transferred into the female's genital openings via the male's copulatory pleopods, the female then stores the spermatozoa in two seminal vesicles; consequently fertilisation is internal (Crothers, 1976; Ruppert & Barnes, 1994). The egg sac (also referred to as the plug or sponge) appears a few months later attached to the oviparous seta of the abdomen; the females bear these eggs for the several weeks prior to hatching (Crothers, 1976). An average female may carry up to 200,000 fertilised eggs (NIMPIS, 2002).

Shortly after hatching the prezoeal larvae undergo a moult and enter the zoeal stage. During this pelagic larval phase the larvae possess two distinctive, elongated spines, one rostral the other dorsal, which are believed to function as a deterrent to predators. If they survive this period as members of the zooplankton the larvae then enter a post-larval stage, the megalopa. During this stage *C. maenas* spends the majority of its time as part of the benthos, but is capable of swimming and may spend periods in the water column. At the end of the post-larval stage the megalops metamorphoses into a juvenile crab. The duration of the larval and megalopa stages is highly variable (lasting anywhere from 17 to 80 days) with the rate of development affected by numerous environmental factors such as temperature, salinity and food availability.

C. maenas typically attain maturity at 2-3 years of age, after multiple moults (NIMPIS, 2002).

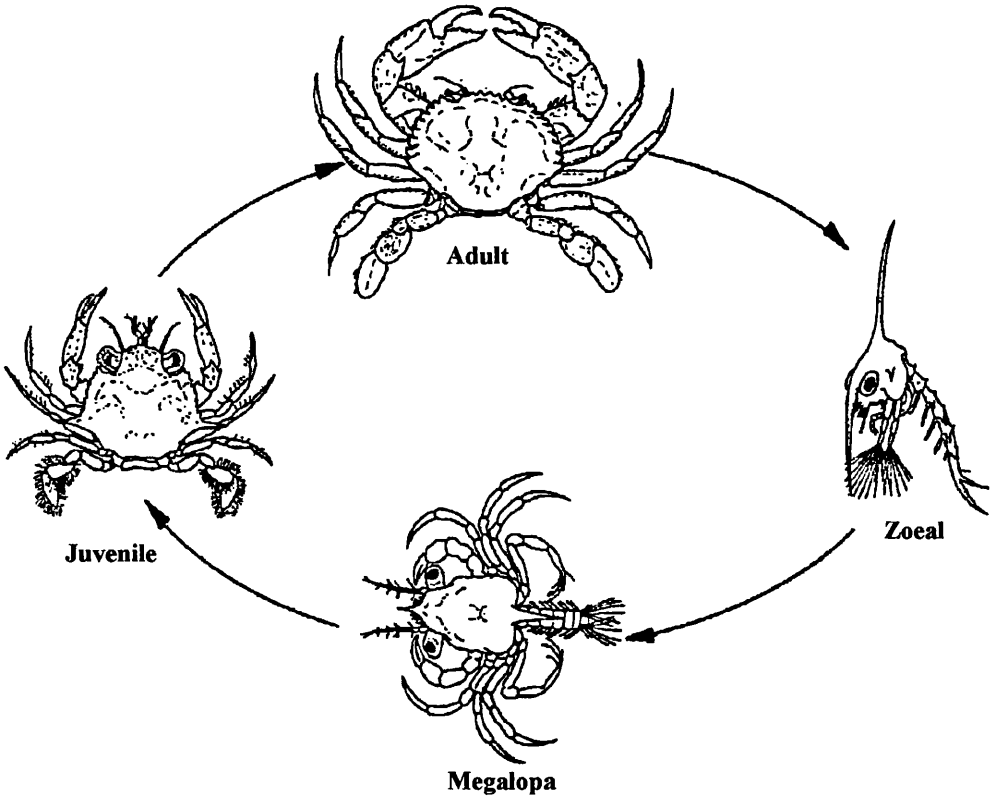


Figure 5. Generalised diagram of the lifecycle stages of a typical brachyuran crab taken from Wickins and Lee (2002). The egg stage and carrying of the egg sac by the female are not shown.

1.3 Species biology – *Litopenaeus vannamei*

L. vannamei is a member of the penaeid shrimps and is highly abundant on muddy substrates extending from the shoreline to a depth of *ca.* 72 m (Dore & Frimodt, 1987). As with the majority of shrimp, *L. vannamei* is a predominantly bottom-dwelling species spending the majority of its time creating shallow excavations in soft substrates. Pacific white shrimp are scavenging detritivores consuming most forms of organic detritus. Wild populations are distributed along the eastern Pacific from Mexico (Sonora) to Tumbes in northern Peru, with the highest population density occurring off the Panamanian coastline (Perez Farfante & Kensley, 1997).

L. vannamei typically requires year round water temperatures in excess of 20°C, but is euryhaline, tolerating salinities from 1 to 40 ppt; consequently, wild populations are restricted to tropical brackish/marine habitats (Allen Davis et al, 2004; http://fao.org/2009).

L. vannamei is currently the most commercially-cultured crustacean species on Earth. It surpassed the Giant tiger prawn, *Penaeus monodon*, as the primary species of shrimp aquaculture in 2004 due to the difficulty of breeding and greater susceptibility to disease of the latter (http://fao.org/2009). Global aquaculture production of *L. vannamei* in 2007 was estimated as *ca.* 2,300,000 tonnes (http://fao.org/2009).

1.3.1 External anatomy of *L. vannamei* (refer to Figure 6)

The Penaeidea, comprising the shrimp and prawns, contains some of the most primitive decapod species (Ruppert & Barnes, 1994). *L. vannamei* possesses a typical shrimp body type, characterised by cylindrical, elongated, laterally compressed thoracic and abdominal regions. The abdomen or pleon, comprising 6 somites or segments and ending in a telson and paddle-like biramous uropods, is well developed and heavily muscled for swimming. The large uropods and powerful abdominal muscles are used to generate the rapid acceleration required for backward escape responses and tail 'flicks' used to evade/discourage predators. Each somite is enclosed by a dorsal tergum and a ventral sternum and the sixth abdominal somite bears the three cicatrices. The five anterior somites each bear a pair of large fringed pleopods which are utilised as the principal swimming organs. The head and thorax are fused forming a cephalothorax exhibiting a large, anterior, keel-shaped rostrum. The rostrum is serrated, bearing 7-10 dorsal teeth and 2-4 (although occasionally as many as 8) ventral teeth. The exoskeleton is thin, flexible and translucent with a bluish hue over much of the animal, due to chromatophores, with higher concentrations near the margins of the telson and uropods (Eldred and Hutton, 1960). The animal has stalked laterally mobile compound eyes as well as an antennula (possessing paired antennules) and a highly elongated pair of antennae. Mouthparts comprise two pairs of mandibles and three pairs of maxipilleds. Five pairs of thoracic limbs or pereiopods are utilised for walking and feeding. The three anterior pairs are chelated (but not enlarged) and are used primarily for food handling, as well as walking and to a lesser extent swimming. At maturity, *L. vannamei* attains a maximum length of 23 cm, with the female commonly larger and faster growing than the male.

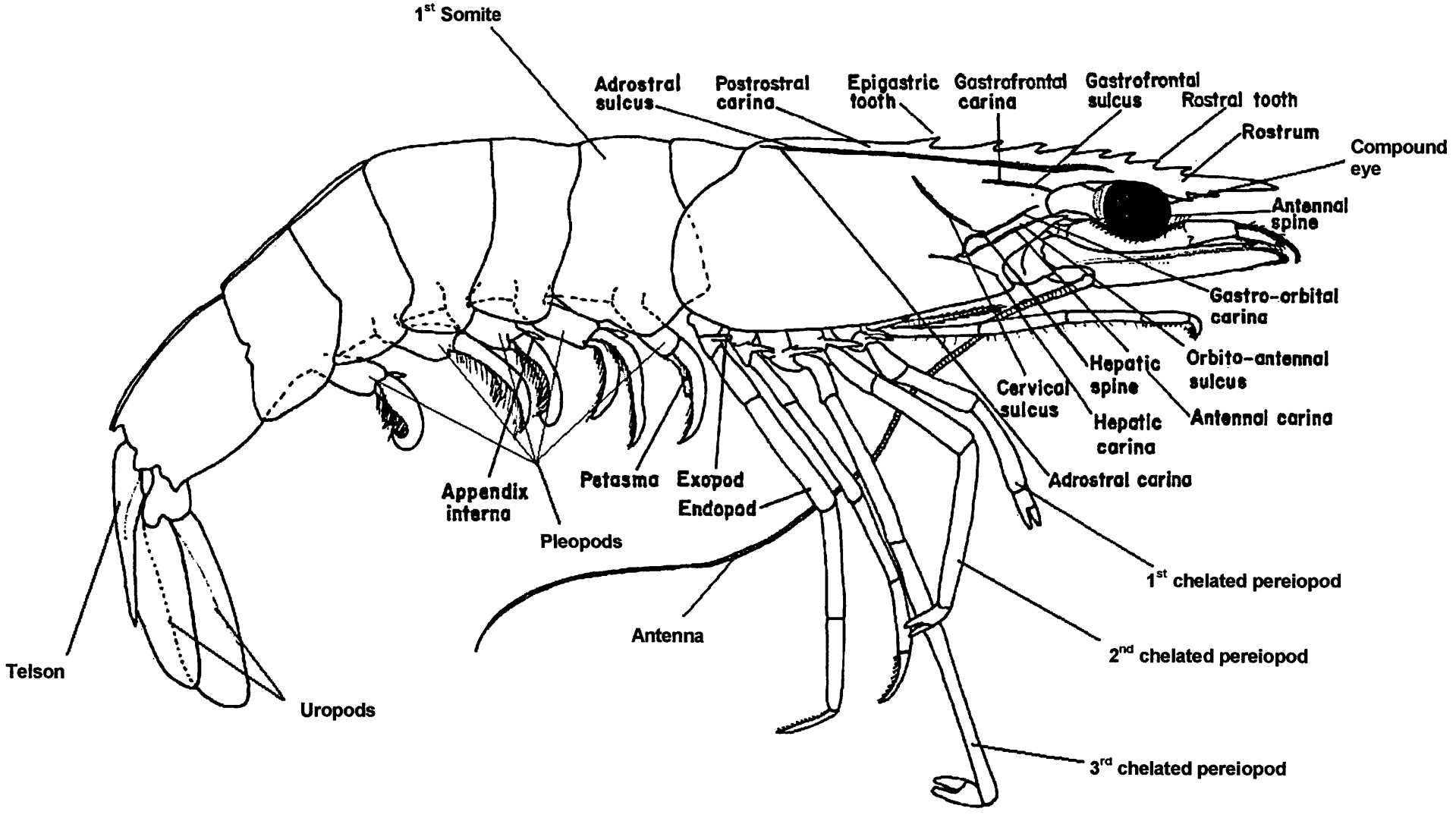


Figure 6. Lateral view of the external features of a typical penaeid shrimp, showing carapace armature and ornamentation (modified from MacLaughlin, 1980).

1.3.2 Internal anatomy of *L. vannamei* (refer to Figure 7)

The general anatomy of the gastrointestinal tract varies little amongst decapod species; consequently the functions performed by the various regions of the alimentary canal in *L. vannamei* are virtually identical to those in *C. maenas* (Section 1.2.2). The gut is again divided into three main regions, the fore, mid and hind guts and begins with an anteroventral mouth and oesophagus. The primary differences lie not in the function of the regions but in their appearance, the fore and mid guts closely parallel those of *C. maenas*. The stomach or 'gastric mill' of shrimp, though not as large or as clearly differentiated into cardiac and pyloric regions as in brachyurans, still possesses multiple chitinous ridges and denticles. The structure and morphology of these ridges and denticles can vary greatly between species and may be modified to varying degrees by diet and food particle size (Pinn et al, 1999). The hepatopancreas remains the animal's primary digestive/storage gland containing R-cells which house the crustacean's glycogen, lipid and element (Cu, Zn, P & S) stores (Al-Mohanna & Nott, 1987). The cellular composition of the penaeid hepatopancreas is the same as that described for *C. maenas* in Section 1.2.2. The organ comprises numerous blind-ending tubules (that empty into the midgut) lined by a cuboidal epithelium comprised of E, F, B and R-cells. In penaeid shrimp F-cells are an intermediate stage between the E and B-cells responsible for the synthesis (and secretion) of zymogen for extracellular digestion during the first 2 h after feeding (Al-Mohanna & Nott, 1989). Consequently, the only major difference between the species is with regard to the organ's gross morphology, in *L. vannamei* it takes the form of a large ovoid organ encasing the mid gut, reddish orange in colour and occupying most of the rear half of the thoracic cavity (MacLaughlin, 1980).

In shrimp the mid and hindguts are highly elongated due to the increased development of the abdomen but perform the same functions as outlined in *C. maenas* (Section 1.2.2). Detail on the gut microbiota of penaeid shrimp is covered in Section 1.4.

The white shrimp's circulatory system is more complex and less open than that of *C. maenas*. The heart, located dorsally within the thorax, pumps haemolymph to the organs and tissues via three major arteries which narrow into a network of arterioles. The arteries/arterioles contain muscular sphincters which provide some control over haemolymph distribution. From the arterioles the haemolymph passes into a capillary-like tubule network. The haemolymph exits this tubule network into tissue sinuses where it bathes the tissues; it then collects in ventral haemocoelic sinuses before being returned to the pericardium via the gills. The vascular system of *L. vannamei* exhibits greater differentiation and efficiency as the animal is far more active than it brachyuran counterpart, utilising rapid bursts of speed to escape predation. As with all crustaceans the total number of circulating haemocytes present in *L. vannamei* is variable, but is *ca.* 2.5 x 10^7 cell ml^{-1}(Chiu et al, 2007). *L. vannamei* possesses the same haemocyte types but with slight variation to the proportions stated for *C. maenas* (granular type, *ca.* 70%; semi-granular type, *ca.* 25%; hyaline, *ca.* 5%; Montero-Rocha et al, 2006).

In common with other penaeid shrimp, *L. vannamei* possesses dendritic or dendrobranchiate gills. The gills possess a central axis that supports numerous secondary laminae giving rise, at right angles, to filaments divided into two branches near their termini (Wu et al, 2009). Each primary filament further divides into secondary filaments. The central axis is anchored to the wall of the cephalothorax via a tubular structure. The ventral margins of the carapace fit loosely against the body wall, allowing water to enter the brachial chamber at any point along the posterior and

ventral carapace edges (Ruppert & Barnes, 1994). The flow of haemolymph through the gill lamellae is unaltered from that outlined in Section 1.2.2, for *C. maenas*. In the female, ovaries are large and elongated extending from the anterior region of the cephalothorax (below the rostrum) into the pleon, with a highly lobed median region in the vicinity of the hepatopancreas (Figure 7). In comparison the testes of the male are considerably smaller and occupy a central position slightly posterior to the hepatopancreas and above the first pair of (copulatory) pleopods.

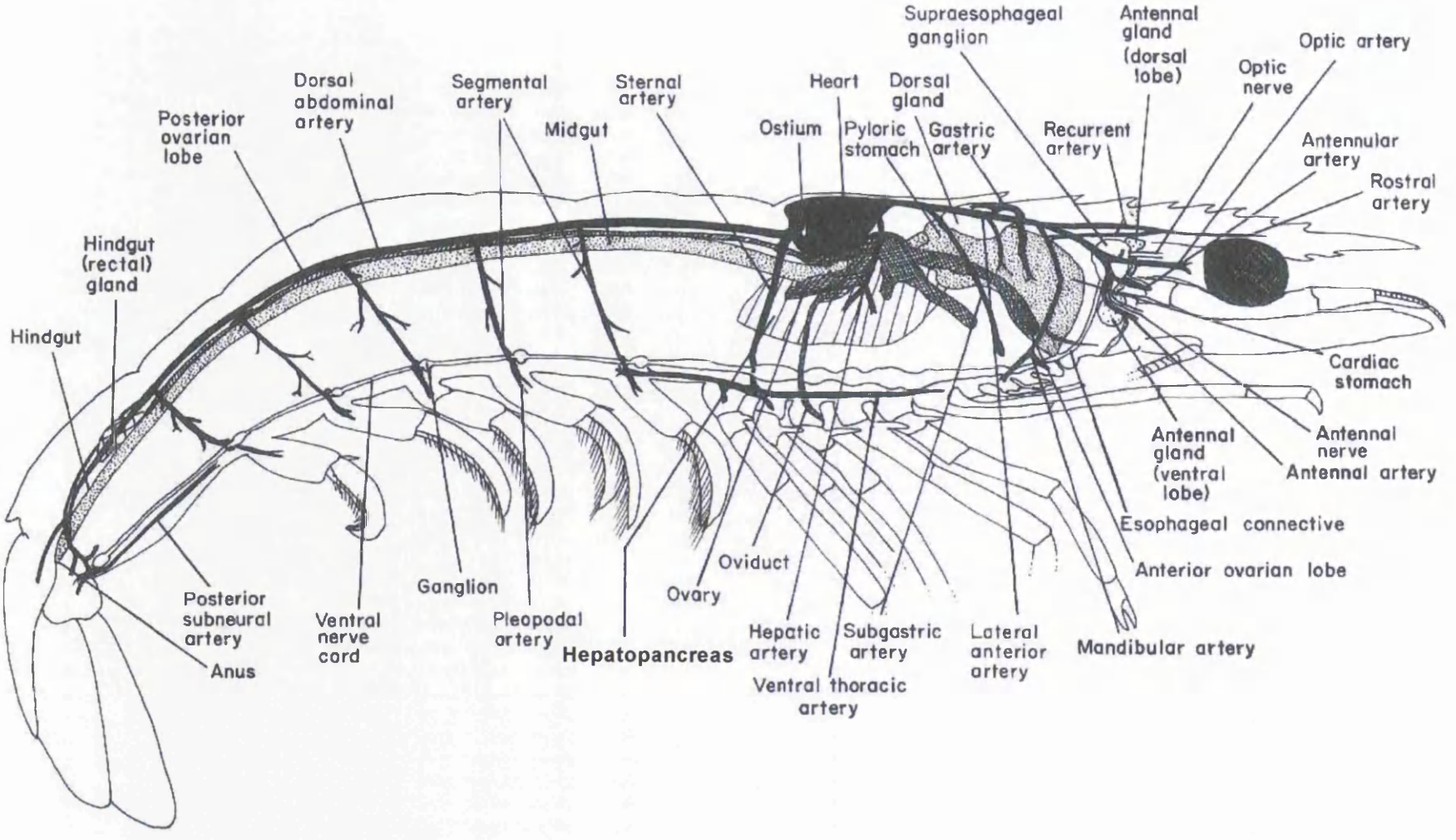


Figure 7. Lateral view of the internal features of a typical penaeid shrimp, displaying circulatory, digestive, nervous and reproductive systems (modified from MacLaughlin, 1980).

1.3.3 Reproduction and life cycle of *L. vannamei* (refer to Figure 8)

Male *L. vannamei* become sexually mature at around 20 g and females from 28 g onwards, typically at an age of 6–7 months. In wild populations, the adult animals live and mate offshore. Breeding has been recorded occurring either throughout the year, or during two distinct seasons, and is instigated by the female via energetic swimming and 'jumping' behaviour, towards the end of the moult cycle. Due to the semi-translucent nature of the animal's carapace it is possible to observe colouration changes in the ovaries in the 24 h prior to mating; from an off white to a golden or greenish brown (Brown and Patlan, 1974). Sperm transfer is perpetrated by an anterior modified (tubular) pair of copulatory pleopods. Copulation occurs with the animal's ventral surfaces oriented parallel to one another, rather than at right angles as is the case in many shrimp species (Dall et al, 1990; Ruppert & Barnes, 1994). Fertilisation is external in penaeid shrimp with the male depositing spermatophores onto the female's thelycum (modified ventral sternal plates on the surfaces of the 7^{th} and 8^{th} thoracic somites) (Dall et al, 1990). *L. vannamei* is as an open-thelycum species; consequently, for successful fertilisation, spermatophore deposition must occur no more than three days (typically only a few hours) prior to the female spawning (Perez Farfante, 1975; Dall et al, 1990). Unlike all other decapod species, penaeid and related shrimp do not carry their fertilised eggs until hatching but shed them directly into the water column. *L. vannamei* weighing 30–45 g will spawn 100,000–250,000 eggs of approximately 0.22 mm in diameter. These eggs typically hatch after 16 h in the water column with the animal referred to as a nauplius (Figure 8). The larvae do not feed during this naupliar stage which lasts 24-36 h, but subsist off their yolk reserves. During this period larval *L. vannamei* undergo six nauplii

stages or instars after which they enter the protozoea stage (Kitani, 1986). This protozoea stage (comprising a further 3 instars) lasts 4-5 days and is followed by a 3-4 day mysis stage (three instars) (http://fao.org/2009). After the mysis stage the animal enters into the post-larval stage (megalops), lasting *ca.* 25 days. During these post-naupliar larval stages (protozoea, mysis and early post-larvae respectively) the animal remains planktonic, consuming phytoplankton and zooplankton, and is carried towards the shore by tidal currents. Approximately 5 days after moulting into post-larvae *L. vannamei* shifts away from planktonic food sources and begins feeding on benthic detritus and infauna, such as worms, bivalves and crustaceans (http://fao.org/2009). The animal remains in this inshore habitat (typically in estuaries, mangroves and lagoons) feeding and growing into a juvenile and then sub-adult and attaining maturity in 180-300 days (Dall et al, 1990). A diagram of the stages of a typical penaeid life cycle can be seen in Figure 8.

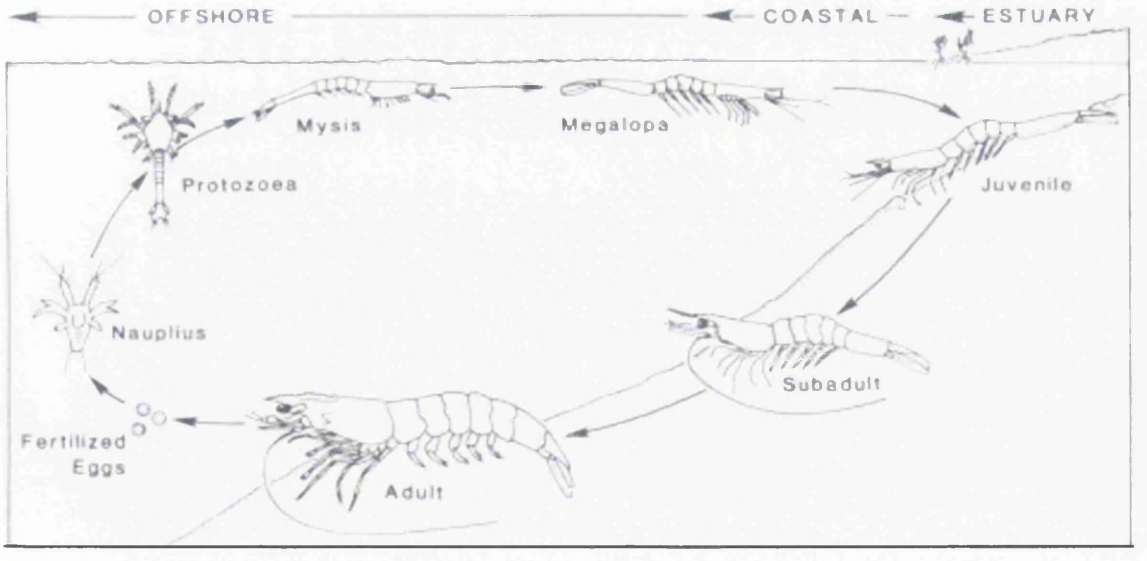


Figure 8. A generalised diagram of the penaeid shrimp life history taken from Bailey-Brock and Moss (1992).

1.4 Gastrointestinal microbiota of decapod crustaceans with particular reference to that of penaeid shrimp

Since no specific information on the compositional/functional significance of gut microbiota in *C. maenas* is currently available, this will be a general discussion of crustacean G.I. microbiology, with specific reference to brachyurans and penaeid shrimps.

The microorganisms present in the crustacean G.I tract at any given time may be ingested transients or resident microbes (Harris, 1993). Determining the taxonomy of bacteria present within the G.I tract of an animal is an extremely difficult proposition, due to the sheer range and differing growth conditions. Relatively little is known about the microbial community composition and its role in the human G.I tract, and even less about that of aquatic invertebrates. However, distinct bacterial communities have been determined as being present throughout the crustacean digestive tract, from the oral region to the hindgut (Harris, 1993; Johnson et al, 2008). Although evidence exists that some Crustacea maintain permanent and consistent G.I microfloral communities, e.g. the prawns *Upogebia africana* and *Callianassa kraussi*, many do not (Harris et al, 1991; Harris, 1993). This would suggest that in many cases aquatic invertebrate G.I microflora is composed primarily of ingested bacteria and is therefore highly variable and transient in nature (Harris, 1993). Bacteria ingested by detritivores such as *L. vannamei* have also been shown to be important as viable sources of nutrition (Lau et al, 2002). Determining whether a microbe is a permanent or transitory member of the G.I microflora is, however, problematic (Peter et al, 2008). G.I microbial community composition will depend heavily on the animal's habitat and diet. A further possible factor responsible for the highly variable nature of crustacean

G.I microbial communities is the shedding of the fore/hindgut lining and exoskeleton during moult (Dempsey et al, 1989). This may be a reason why many crustaceans consume their moults, i.e. in an attempt to re-introduce a stable, autochthonous, residential gut flora as well as reabsorbing nutrients (Dempsey et al, 1989; Oxley et al, 2002). The presence of such a stable residential gut flora was noted by Dempsey et al (1989) in the hindgut of penaeid shrimp. Previous studies indicate that the highest bacterial densities are supported by the posterior region of the G.I tract (mid & hindgut) (Gomez-Gil et al, 1998; Oxley et al, 2002). This is likely due to the disruptive grinding and filtering actions of the foregut, which also possesses a highly ornamented, chitinous lining, all of which would likely preclude any substantial bacterial colonisation (Oxley et al, 2002). The hindgut would also be more amenable to microbial colonisation due to the lack of any active digestion in the region (i.e. lower levels of enzymes, surfactants and sloughing) and the presence of high concentrations of substrates and nutrients (i.e. remaining products of digestion) (Lau et al, 2002).

The assessment of gill/gut content bacteria from seven Japanese coastal crustacean species (6 of them brachyurans) indicated that the vast majority (87%) of the strains present (both aerobic & facultative anaerobes) were members of the Vibrionaceae (Sugita et al, 1987). In addition the majority of isolated strains were anaerobic; 1024 out of 1564 (Sugita et al, 1987). That the six Japanese crab species shared a similar habitat type and lifestyle with *C. maenas* is an indicator that a large portion of this species bacterial (gut) flora likely comprises *Vibrio* spp. and related forms. In addition, it appears that the microbial community present in the gut of penaeid shrimp is composed of several types, but dominated by only one or two genera (Dempsey et al, 1989; Moss et al, 2000; Oxley et al, 2002). In the case of *L. vannamei*, the aerobic

culturable G.I microbiota is dominated by *Vibrio* and *Aeromonas* spp. (Vandenberghe et al, 1999; Moss et al, 2000; Johnson et al, 2008) further supporting the findings of Sugita et al (1987).

In conclusion, it is likely that the culturable G.I bacterial community of penaeids (and indeed crustaceans generally) is composed of a subgroup of ingested bacteria that predominate due to a tolerance of the physiochemical conditions found in the gut (Dempsey et al, 1989; Moss et al, 2000; Lau et al, 2002). This is further indicated by the significantly lower species diversity of the hindgut microbial community of cultured *L. vannamei* when compared to that of the grow-out water (Johnson et al, 2008). The composition of this subgroup will vary, both between individual crustaceans and species, due to numerous factors. In *L. vannamei* such factors likely include; salinity, temperature, habitat (i.e. substrate), lifecycle stage, moult cycle stage, health status, gut passage time and diet (Dempsey et al, 1989; Vandenberghe et al, 1999; Moss et al, 2000; Sakami et al, 2008). A picture of overall bacterial community structure in aquatic invertebrates is currently unavailable as a large portion of natural bacterial communities are unculturable (Zengler et al, 2002; Sakami et al, 2008). Culture-independent molecular techniques, such as denaturant gradient gel electrophoresis (DGGE) are, however, starting to be used to examine bacterial community diversity and composition in relation to invertebrates, but with the emphasis on habitat bacterial populations rather than gut microbiota (Zengler et al, 2002; Sakami et al, 2008).

1.5 Aquaculture with particular reference to that of shrimp

Aquaculture is defined as the farming of both freshwater and saltwater aquatic organisms including finfish, molluscs, crustaceans and aquatic plants (http://fao.org/2009). Unlike fishing, aquaculture implies the cultivation of aquatic populations under controlled, typically intensive, conditions. Such cultivation requires intervention in the rearing process, to varying degrees, to enhance production. This intervention may take the form of regular stocking, feeding, immunisation against disease, treatment for parasites, protection from predators etc.

Aquaculture is not a recent concept; finfish culture was recorded in China as early as 2,500BC, but it has only been regarded as a commercially significant source of aquatic organisms since the 1960's. The contemporary rise in popularity of aquaculture has stemmed from the increasing scarcity and consequently price, of wild caught animals. The peaking of capture rates and subsequent decline in wild stocks, coupled with an increasing demand from a growing human population has led commercial aquaculture to exist on an unprecedented scale.

Aquaculture continues to be the fastest growing animal food-producing sector and accounted for 47% of the world's (food) fish supply in 2006 (FAO SOFIA Report, 2008). Aquaculture production reached 45.7 million tonnes by weight and $56.5 billion by value in 2000. By 2006, this had increased to 51.7 million tonnes by weight and $78.8 billion by value (FAO SOFIA Report, 2008). Worldwide, aquaculture has increased at an average compounded rate of 9.2% per year since 1970, compared with only 1.4% for capture fisheries and 2.8% for terrestrial farmed meat production systems. As of 2000, just over half of global commercial aquaculture (50.3%) was of marine species, referred to as mariculture; the reminder was divided between

freshwater and brackish water sources (45.1% and 4.6%, respectively) (Tacon, 2003). The vast majority of these aquatic animals/plants are cultivated for human (or occasionally animal) consumption; however, some species are required for other purposes (e.g. ornamental). The primary focus of production of the three areas of aquaculture, both financial and by weight figures is the farming of finfish species. As of 2006, finfish comprised 63% of global aquaculture production, worth over $47 billion. In contrast the production of crustaceans made up only 9%, but was worth over $18 billion (FAO SOFIA Report, 2008). Crustacean aquaculture has exhibited the largest increase in annual growth of any of the major species groups, both in the last 8 years (an average of 16% per annum) and overall since 1970 (an average of 18% per annum) (FAO SOFIA Report, 2008).

Global crustacean aquaculture is dominated by penaeid (shrimp/prawn) farming, the annual production of which far exceeds that of wild capture fisheries, with over 70% of the shrimp produced globally sourced from aquaculture. Consequently, the commercial culture of penaeid shrimp is a massive global industry worth many billions of dollars annually. Several species of shrimp are cultured worldwide, these include the Western blue shrimp (*Penaeus stylirostris*), the Chinese white shrimp (*Penaeus chinensis*), the Kuruma shrimp (*Penaeus japonicus*), the Indian white shrimp (*Penaeus indicus*) and the Banana shrimp (*Penaeus merguiensis*). However, over 80% of the global shrimp aquaculture market is made up of only two species, the Giant tiger prawn, *Penaeus monodon* and the Pacific white shrimp, *Litopenaeus vannamei*. *P. monodon* dominated penaeid aquaculture (i.e. was the main species cultivated) until 2004 when it was surpassed by *L. vannamei*. Despite *L. vannamei*'s smaller size at maturity (compared to the Giant tiger prawn) it exhibits greater yields and, therefore, profit, due to its greater fecundity and resistance to disease

(http://fao.org/2009). The FAO lists the total aquaculture production for *L. vannamei* in 2007 at 2,296,620 tonnes worth an estimated $8,815,854,000. The Pacific white shrimp is commercially cultured in 30 countries, with the five largest producers listed in Table 1.

In the 1970s a shift in shrimp farming methods occurred, from subsistence style farming toward the more intensive practices of an export-oriented business. This began with the use of 'extensive' shrimp farms, these compensated for low yield per area (*ca.* 25,000 animals/ha) with increased pond sizes; instead of ponds of just a few hectares, pond sizes up to 100 ha (one km^2) were used. Technological advances allowed the development of semi-intensive (*ca.* 100,000–300,000 animals/ha), intensive farms (*ca.* 300,000-1,200,000 animals/ha) and super-intensive farms (<1,200,000 animals/ha); with animals raised on formulated pellet feeds and ponds closely managed (Rosenberry, 2004). Until the mid 1980s all shrimp farms were stocked with wild caught post-larvae. However, when depletion of wild stocks was detected the industry began raising shrimp from eggs and maintaining adult shrimp for reproductive purposes (i.e. broodstock) in specialised hatcheries. Hatcheries can vary greatly in scale, but the purpose of all remains the maintenance of broodstock and raising of post-larval shrimp from fertilised eggs. Although some farms introduce post-larvae directly into ponds, typically, most possess 'nurseries' where the post-larvae are housed, for approximately 3 weeks, until they have grown into juveniles. The juvenile shrimp then enter the 'growout' phase where they are transferred to large open ponds and fed until they reach a marketable size (or maturity if required for broodstock). This growout phase has a typical duration of 4-6 months.

Table 1. FAO aquaculture production figures (gross weight in tonnes) of *Litopenaeus vannamei*, for the top five producing nations in 2006 (FIGIS; http://fao.org/2009).

	Production, rounded (tonnes)
China	1,242,000
Thailand	501,000
Vietnam	349,000
Indonesia	326,000
India	132,000

The shrimp aquaculture industry has been recently developing closed or re-circulation shrimp production systems, with low or zero water-exchange, in order to reduce disease and control effluent. However, such systems are expensive and a high proportion of farms, particularly in poorer nations, still utilise the extensive/semi-intensive methods. This intensive type of shrimp farming is unsustainable long-term and may cause significant ecological harm. There is a great deal of evidence to indicate the perpetration of damage to mangroves, mud flats, salt marshes and other forms of coastal wetland (World Bank, NACA, WWF and FAO, 2002). It is estimated that Thailand alone has lost 83.7% of original mangrove since 1975 (Thornton et al, 2003). A further negative impact from such shrimp farming, experienced by Thailand, is the salinisation, pollution (through run off) and depletion of supplies of fresh ground water (EJF, 2004). Even in the more intensive systems, it is estimated that 30% of a farm's pond water volume is exchanged daily (EJF, 2004). In Thailand, shrimp farms have been reported to discharge approximately 1.3 billion cubic metres

of effluent annually (World Resources Institute, 1998/99). In coastal areas sporting a high density of such poorly managed open pond systems, effluent run off can negatively impact on marine ecosystems. Even in well managed semi-intensive/intensive systems, prolonged use of a pond leads to an incremental build-up of organic sludge at the pond's bottom from accumulated waste products (e.g. dead animals and unconsumed feed) and excrement. Flushing alone is insufficient to fully dislodge this sludge and unless it is removed via mechanical means the pond will eventually have to be abandoned (typically after 2-3 years). Such irresponsible shrimp aquaculture leaves behind a wasteland with the soil rendered unusable for any other purpose due to the high levels of salinity, acidity, and potentially toxic chemicals. Thus, the ability to maintain good water quality and high feed conversion rates is vitally important for two reasons; firstly to reduce environmental impact and secondly to enhance animal health/growth and subsequently yield. Substantial improvements in both of these can be accomplished via more efficient farm management and animal husbandry. However, the significance of beneficial microorganisms within the growout water cannot be ignored (Maeda & Chiu-Liao, 1992; Ma et al, 2009; Wang & He, 2009)

Currently, however, the most significant problem facing modern shrimp aquaculture is the prevalence and control of disease (Skjermo & Vadstein, 1999). The majority of pathogenic infections suffered by cultured larval and post-larval *L. vannamei* are bacterial in nature; principal amongst these is luminous vibriosis, the causative agents being *Vibrio* spp. such as *Vibrio harveyi* and *V. campbellii* (Selvin & Lipton, 2003; Jayasree et al, 2006; Soto-Rodríguez et al, 2006). *Vibrio nigripulchritudo*, is responsible for outbreaks of so-called 'Summer syndrome' afflicting *L. vannamei* farms in New Caledonia (Lemonnier et al, 2006; Castex et al, 2008). As well as

bacterial pathogens, *L. vannamei* is also highly susceptible to viral infections, chiefly Whitespot syndrome; a highly lethal infection capable of wiping out entire farms in a matter of days (Phuoc et al, 2009). Taura syndrome, also viral, was principally restricted to *L. vannamei* farms in the Americas, but has since become a global problem (Lotz et al, 2005; Dhar & Allnutt, 2008). The explosion in popularity of *L. vannamei* for aquaculture has contributed significantly to this problem; the worldwide demand for and transportation of larvae and broodstock has resulted in the rapid dissemination of these diseases (Dhar & Allnutt, 2008).

Since most diseases (particularly viral) suffered by cultured shrimp often cannot be identified quickly enough to be treated effectively, the industry's efforts are focused primarily on the prevention of disease outbreaks. The various approaches to disease control in shrimp aquaculture, as well as their benefits and drawbacks, are discussed in detail in Section 1.6.3.

1.6 Probiotics

Probiotics are defined by Fuller (1989) as "live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance." This definition incorporates three principal tenets, firstly, that the supplement is microbial in nature; secondly, that it is delivered via feed and, thirdly, that it is live at the time of administration. Fuller's is still regarded by most in the field as the 'true' definition of a probiotic. However, with an increasing public profile and subsequent popularity of probiotic products, the term 'probiotic' has been used to describe supplements and techniques outside the scope of Fuller's definition. For example in aquaculture the addition of bacterial strains to growout water (as opposed to feed) to improve water quality and thus animal survival, has been described as probiotic, when in actuality it is bioremediation (Harris, 1993). In addition, the aspect of the administration of live micro-organisms has also been challenged by the description of killed bacteria and microbial products as probiotics (Díaz-Rosales et al, 2006). Whether the definition will be expanded to incorporate the aforementioned under the term probiotic is uncertain, but as far as this project is concerned the term probiotic is used as defined by Fuller (1989).

1.6.1 History of probiotics

The term probiotic is derived from the Greek "pro" meaning for and "bios" meaning life. Although probiotics have been consumed for centuries as natural components in health-promoting foods (e.g. natural yogurt, cheeses, sour cream, fermented milk and cured meats) they were typically only present as a by-product of fermentation or

preservation techniques (Garaiova & Muchová, 2008). Observation of the significance of the role played by strains of bacteria in the digestive system and the potential of modifying the gut flora to replace harmful strains with beneficial ones was first made by Ukrainian scientist and Nobel laureate, Eli Metchnikoff in 1907 (Gibson & Fuller, 2000). Metchnikoff believed that the ageing process was speeded up by compounds produced by proteolytic bacteria such as *Clostridia* spp. present in the gut, a process he termed the "autointoxication effect". He hypothesised that administering harmless lactic-acid bacteria (via fermented milk) would decrease the intestinal pH and that this in turn would suppress the growth of such proteolytic microorganisms. Metchnikoff isolated the bacteria present in sour milk products after observing that the Bulgarian peasants who consumed such products exhibited noticeably greater longevity. The lactic acid bacterium isolated was *Lactobacillus bulgaricus* (referred to at the time as 'Bulgarian bacillus') and was consumed daily by Metchnikoff until his death in 1916 at the age of 71. In the 1930's, after the discovery that *L. bulgaricus* did not survive transit of the digestive tract, research was undertaken involving lactic acid bacteria isolated from human intestinal sources. This led to the isolation of *Lactobacillus acidophilus*, which was utilised in a commercial fermented milk product in the United States (Rettger et al, 1935). The work of Metchnikoff also inspired the research of a Japanese microbiologist, Minoru Shirota, who isolated possibly the most widely known probiotic bacterium, *Lactobacillus casei* strain Shirota (Garaiova & Muchová, 2008). *L. casei* Shirota is utilised in a fermented milk drink produced by the Japanese food company Yakult Honsha Ltd., who were the first to commercialise the sale of live bacteria in a fermented milk drink for human consumption in 1935. In the 1960's live microbial products became popular in animal agriculture as an alternative to the widespread, prophylactic use of antibiotics (Fuller, 1989). What followed was a

natural progression to the present day where live microbial feed supplementation is common place. Included within this progression were three key observations; that germ-free animals were more susceptible to infection than their conventional counterparts; that oral antibiotics actually increased an animal's susceptibility to infection; and that the administration of faecal enemas may be useful in the control antibiotic-associated diarrhoea (Gibson & Fuller, 2000).

Characteristics required in probiotic bacterial strains include; a lack of pathogenicity or toxicity, a resistance to conditions encountered within the host organisms G.I. tract and the capacity to induce a beneficial effect in the host. Other less vital characteristics include the ability to adhere to intestinal mucus/cells, the facility to remain viable during transport/storage and to adapt to the commensal or indigenous microbiota (Gorbach, 2002).

1.6.2 Potential of probiotics to improve health status in terrestrial vertebrates (primarily humans)

Public opinion has long held probiotic supplementation to be beneficial to the health of humans and animals. However, these benefits, such as increased survival, reduced occurrences of disease, etc, are often overemphasised and difficult to attribute accurately due to the nature of *in vivo* testing in humans. Although there has been much investigation into *in vitro* activity the information regarding the *in vivo* mechanisms by which the oral administration of non-pathogenic bacteria may bolster the health status of a host is generalised and incomplete (Fuller, 1991; Gibson & Fuller, 2000; Dunne et al, 2001; Kopp-Hoolihan, 2001).

Intestinal bacteria in terrestrial mammals number in the trillions, with the gut microbiota evolving in symbiosis with the host (Salminen et al, 1998). The majority of research into the relationship between gut microbiota and host organism has been conducted in terrestrial vertebrates, principally humans. Consequently, this section will focus on the efficacy of probiotic administration in humans. The potential of probiotics in aquaculture is outlined in Section 1.6.3.

Although no consensus exists as to what constitutes an "ideal" G.I. microbiota in humans, such a microbiota is regarded as one predominated by saccharolytic (carbohydrate fermenting) microorganisms such as bifidobacteria and lactobacilli (Figures 9 & 10) (Blaut, 2003; Anon (Yakult Ltd.), 2008). The majority of current probiotic bacterial strains are members of these genera, however, other bacteria including members of the Bacillaceae and Enterococci are also utilised (Table 2). There are several potential means, or 'modes of action' by which administration of a probiotic may convey a benefit to the host, as outlined in Table 3 and Figure 11. Recent research on the molecular biology and genomics of lactobacilli has focused on interactions with the immune system and the potential of these micro-organisms as biotherapeutic agents (Ljungh et al, 2009). The potential of these microorganisms in preventing colon cancer and as treatments for antibiotic-associated diarrhoea, travellers' diarrhoea, pediatric diarrhoea, inflammatory bowel disease and irritable bowel syndrome is also under investigation (Ljungh et al, 2009).

Irritable bowel syndrome (IBS) is an umbrella term used to describe a wide and varied range of functional intestinal disorders and should not be confused with inflammatory bowel disease (IBD) which involves the immune system (Baumgart & Carding, 2007; Jones & Lydeard, 2009). IBD encompasses several conditions including; Crohn's disease, diverticulitis, pouchitis and ulcerative colitis. Symptoms of these typically

manifest as pain, diarrhoea, weight loss, tiredness and blood or mucus in the stool. Crohn's disease and ulcerative colitis often present with extra-intestinal manifestations such as skin, liver & eye problems and arthritis; in addition, sufferers also have an increased risk of developing colorectal cancer (Greenstein et al, 1976; Hamilton, 1985).

Table 2. Microorganisms commonly used commercially in probiotic products.

***Lactobacillus* spp.**	***Bifidobacterium* spp.**	**Others**
L. acidophilus	*B. adolescentis*	*Bacillus clausii*
L. brevis	*B. animalis* subsp. *lactis*	*Bacillus coagulans (L. sporogenes)*
L. casei	*B. bifidum*	*Enterococcus faecalis*
L.crispatus	*B. breve*	*Enterococcus faecium*
L. delbrueckii subsp. bulgaricus	*B. infantis*	*Escherichia coli* Nissle 1917
L. fermentum	*B. longum*	*Pediococcus acidilactici*
L. gasseri		*Saccharomyces cerevisiae* var. *boulardii*
L. helveticus		*Streptococcus thermophilus*
L. johnsonii		
L. paracasei		
L. plantarum		
L. reuteri		
L. rhamnosus		
L. salivarius		

From Garaiova & Muchová, (2008)

Despite relatively little research on probiotics as a treatment for the active disease (IBD is primarily treated with mesalamine (a derivative of salicylic acid) and steroids; Rachmilewitz, 1989; Brignola et al, 1994) some trials have reported benefits from the consumption of probiotics (e.g. McFarland & Dublin, 2008). *Escherichia coli* Nissle 1917 was reported as effective in maintaining the remission of ulcerative colitis

(Kruis et al, 2004). Combined probiotic treatments have also been shown to help in cases of diverticulitis (Tursi, 2007).

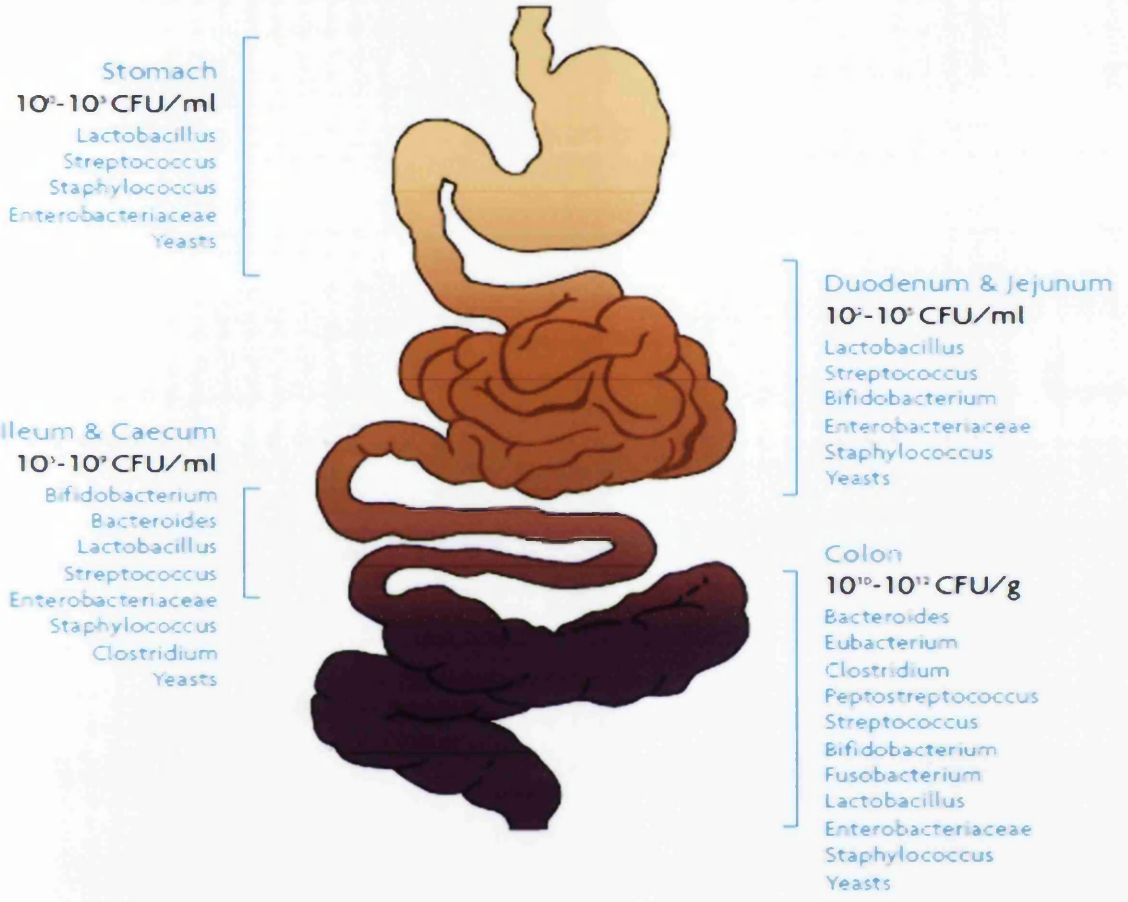


Figure 9. Typical microflora found in the human gastrointestinal tract (from Blaut, 2003).

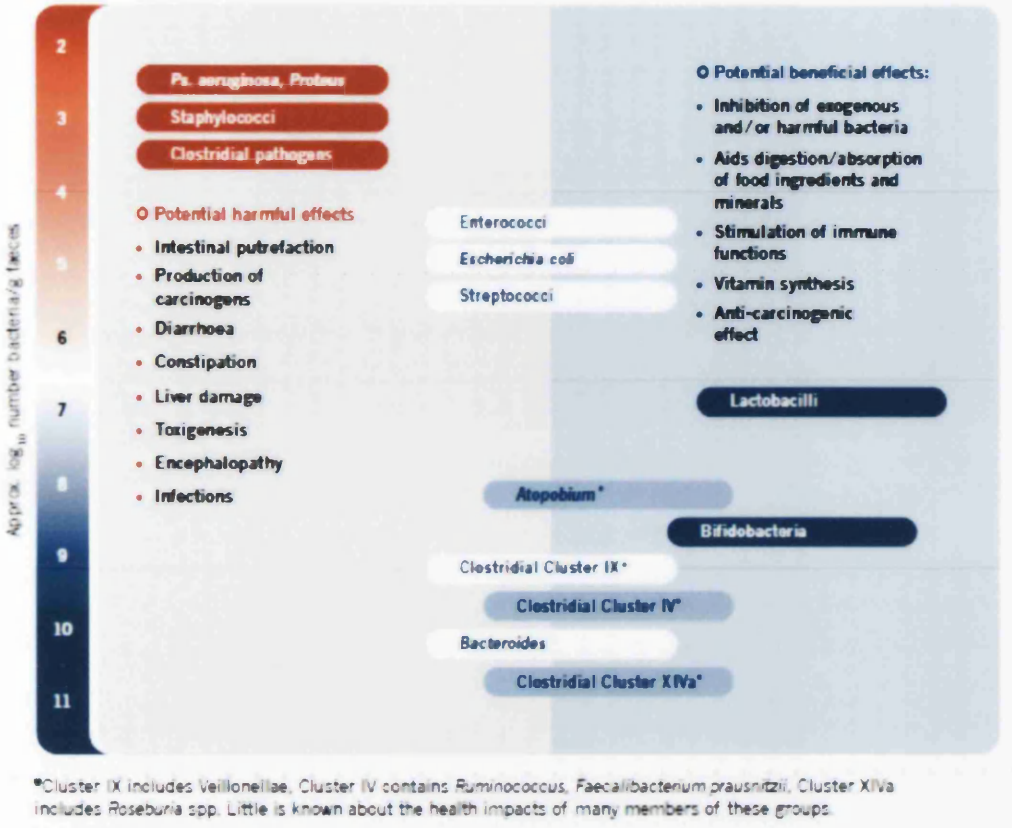


Figure 10. The colonic microbiota and its influence on health (from Anon, 2008 'Probiotics in health and disease' a scientific information report distributed by Yakult Ltd.)

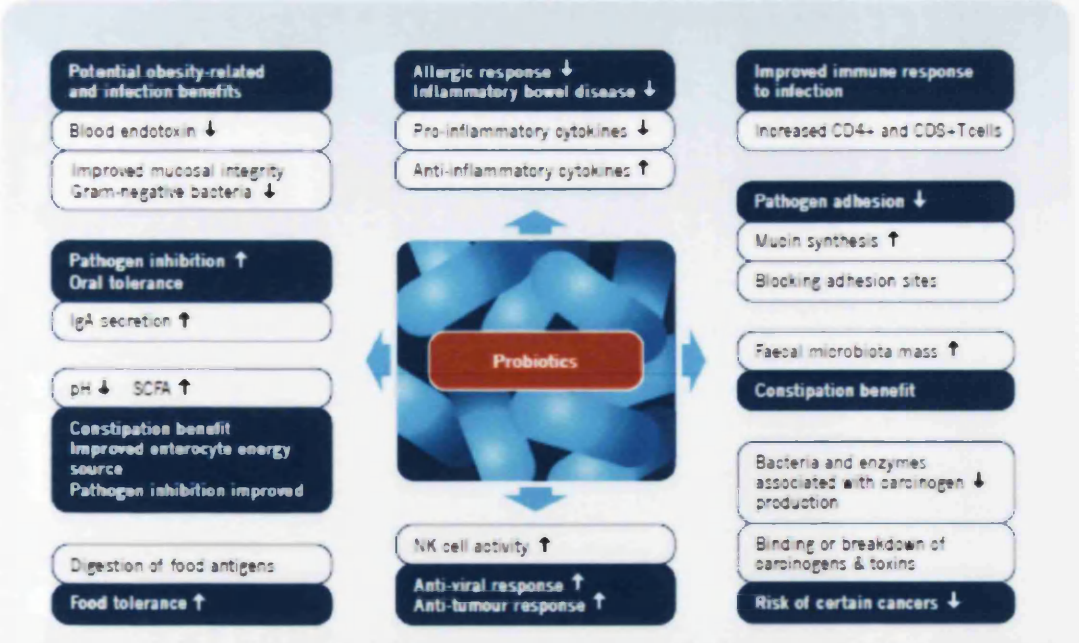


Figure 11. Probiotic mechanisms of activity (evidence is, however, strain-specific and not necessarily conclusive). (From Anon, 2008 'Probiotics in health and disease' a scientific information report distributed by Yakult Ltd.)

Mode of action	Description	Probiotic strain	Target host/test method	Reference
Immune stimulation/ Immunomodulation	Stimulation or alteration of the status of the humoral and/or cellular immune response of a host	*Lactobacillus plantarum*	human	McCracken et al, 2002
		Lactobacillus plantarum	mouse	Perdigon et al, 1999
		Lactobacillus bulgaricus	human	Meyer et al, 2007
		Lactobacillus casei DN 114 001	human	Meyer et al, 2007
		Streptococcus thermophilus	human	Meyer et al, 2007
		Lactobacillus rhamnosus	salmonid fish	Panigrahi et al, 2005
		Bacillus subtilis	human	Duc et al, 2004
		Bacillus clausii	human	Urdaci et al, 2004
		Carnobacterium maltaromaticum B26	salmonid fish	Kim & Austin, 2006
		Carnobacterium divergens B33	salmonid fish	Kim & Austin, 2006
Competitive exclusion principle	Exclusion of potentially harmful microbial species, via competition for i.e. adhesion sites, nutrients (e.g. iron), oxygen, etc.	*Lactobacillus plantarum*	human	Kingamkono et al, 1999
		Bacillus subtilis	fowl	La Ragione et al, 2001
Anti-microbial activity	Inhibition of potential pathogens via production of anti-microbial compounds such as lactic acid, H_2O_2 and bacteriocin-like inhibitory substances (BLIS)	*Lactobacillus plantarum* NCIM 2084	*in vitro* study	Suma et al, 1998
		Pediococcus acidilactici NCIM 2292	*in vitro* study	Jamuna & Jeevaratnam, 2004
		Bacillus coagulans	*in vitro* study	Hyronimus et al, 1998
		Vibrio harveyi	*in vitro* study	Prasad et al, 2005
Enhancement of host digestive performance	Improvement of the host organisms digestive efficiency and thus growth performance (FCR) via production of enzymes, nutrients & other useful substances (e.g. Proteases, amylases, chitinases and vitamins such as B_{12})	*Carnobacterium maltaromaticum*	salmonid fish	Robertson et al, 2000
		Bacillus spp.	cyprinid fish	Yanbo & Zirong, 2006
		Bacillus cereus var. *toyoi*	fowl	Homma & Shinohara, 2004
		Bacillus cereus CIP 5832	pigs	Alexopoulos et al, 2001
Antiviral activity	Mechanism unknown, most work conducted on salmonid viruses - infectious haematopoietic necrosis virus (IHNV) & Oncorhynchus mason virus (OMV)	*Pseudomonas* sp.	*in vitro* study	Kamei et al, 1988
		Aeromonas sp.	*in vitro* study	Kamei et al, 1988
		Vibrio sp.	*in vitro* study	Direkbusarakom et al, 1998
		Lactobacillus casei	mouse	Yasui et al, 2004
Environment Improvement (aquaculture only)	Improvement in growout water quality due to enhancement of microflora resulting in a reduction in nutrient loading (e.g. of organic & inorganic nitrogen sources)	*Lactobacillus* spp. (JK-8 & JK-11)	*in vitro* study	Ma et al, 2009
		Bacillus sp.	*in vitro* study	Wang & He, 2009
		Nitrosomonas sp.	*in vitro* study	Wang & He, 2009
		Nitrobacter sp.	*in vitro* study	Wang & He, 2009
		Lactobacillus sp.	*in vitro* study	Wang & He, 2009

Although IBS does not, in most cases, lead to more serious complications (unlike IBD) it is often a source of great discomfort and embarrassment to sufferers. IBS is characterised by chronic abdominal pain, discomfort, bloating, and alteration of bowel habits (diarrhoea or constipation may predominate) and is suffered by 10-20% of the UK population (Whorwell, 2009). The administration of *Lactobacillus plantarum*, *L. rhamnosus*, *L. casei* Shirota, *L. salivarius* and *Bacillus infantis* has been shown to alleviate symptoms of IBS (Niedzielin et al, 2001; Koebnick et al, 2003; O'Mahony et al, 2005; Matsumoto et al, 2006; Whorwell et al, 2006; Gawronska et al, 2007).

Probiotics have, since their development, been utilised in the treatment of diarrhoea (particularly in children) through support of the commensal microbiota (McFarland, 2006). Several probiotics (including *Saccharomyces boulardii*, as well as mix of *L. acidophilus* and *Bifidobacterium bifidum* and a commercial product containing *L casei DN-114001, L. bulgaricus, and Streptococcus thermophilus*) have been recorded as having significant efficacy in preventing travellers', antibiotic-associated and *C difficile*-associated diarrhoea (Hickson et al, 2007; McFarland, 2007).

Several animal and clinical trials have indicated that lactic acid bacteria may exhibit potential anticancer effects. Lactic acid bacteria are capable of perpetrating beneficial alterations in intestinal physiological conditions within host organisms, e.g. lowering of faecal pH (Biasco et al, 1991). Administration of *L. acidophilus* and *L. casei* resulted in decreased levels of faecal and urinary mutagenicity in patients suffering colon cancer (Lidbeck et al, 1991; Hayatsu & Hayatsu, 1993). Such anti-mutagenic effects are believed to be due to the ability of lactic acid bacteria to bind with heterocyclic amines, which are carcinogenic substances present for example in cooked meat and alcohol (Wollowski et al, 2001). *L. rhamnosus* GG, *L. casei* Shirota and *L. acidophilus* have all been shown to decrease the activity of enzymes β-glucuronidase

and nitroreductase, both thought to be responsible for carcinogenic activity (Goldin & Gorbach, 1984; Ling et al, 1994; Spanhaak et al, 1998). However, just how these results pertain to using probiotics in the reduction of incidences of colon cancer in the wider human population is unclear, as considerable further research is required.

The bacterium *Helicobacter pylori* inhabits various areas of the stomach and duodenum. In approximately 20% of those harbouring *H. pylori*, the bacterium is responsible for a chronic low-level inflammation of the stomach lining and is strongly linked to the development of duodenal and gastric ulcers and stomach cancer (Sipponen & Hyvärinen, 1993). Probiotic administration has been investigated both as a preventative measure and as a treatment. Patients who received *L. casei* Shirota over a three week period displayed significant inhibition of *H. pylori* growth compared to the control group (Cats et al, 2003).

The vital role played by the gastrointestinal microbiota in the general health and well-being of a host organism has, until recently, been considerably underestimated. Despite the widely held conviction that a good diet is vital to good health, the general public appears largely oblivious to the importance of the trillions of microorganisms that inhabit their G.I. tract. However, a new-found public awareness has started to grow from the marketing of probiotic products. Whether probiotics convey much (if any) benefit to individuals possessing a functional, healthy intestinal microbiota is unknown. What is in little doubt, however, is the benefit they provide to those individuals suffering from a wide range of gastrointestinal disruptions and diseases.

1.6.3 Disease control in aquaculture: probiotics, immunostimulants and vaccines

The bulk of research into the efficacy of probiotic administration in aquaculture has focused on their potential benefits to finfish production, principally salmonid species (Ringø & Gatesoupe, 1998; Irianto & Austin, 2002; Balcázar et al, 2006; Kesarcodi-Watson et al, 2008). Fish, despite being 'lower' vertebrates, possess an adaptive specific immune system similar in function to 'higher' vertebrates (Nakanishi et al, 1999). The premise that probiotics should convey a wellness benefit in finfish aquaculture was therefore accepted relatively quickly. Fish, however, (particularly marine species) drink constantly for osmoregulation and this high flow of water through their G.I. tract ensures their intestinal microbiota is far more transitory in nature compared to terrestrial organisms (Cahill, 1990; Spanggaard et al 2000). This is supported by studies indicating that probiotics do not colonise the G.I. tract of fish and only persist for short periods after the cessation of administration (Jöborn et al, 1997; Gatesoupe, 1999; Robertson et al, 2000). Despite this observation, there is clear evidence of the benefits of probiotic administration on the health, survival and growth of cultured finfish. The administration of a *Bacillus* sp. to the common carp, *Cyprinus carpio*, resulted in a considerable increase in growth and digestive enzyme activity (Wang & Zirong, 2006). The feeding of a *Carnobacterium* sp. to Atlantic salmon (*Salmo salar* L.) fry and fingerlings resulted in a decrease in susceptibility to infection by *Aeromonas* spp. and *Vibrio* spp. (Robertson et al, 2000). *C. maltaromaticum* and *C. divergens* have been shown as beneficial to Rainbow trout (*Oncorhynchus mykiss*, Walbaum) in resisting infection by salmonid pathogens *A. salmonicida* and *Yersinia ruckeri* (Kim & Austin, 2006).

A significant aspect which must be addressed when considering the application of potential probiotics for use in crustaceans (indeed all invertebrates) is the lack of any of the gut associated lymphoid tissue (GALT) as present in vertebrates (Smith & Chisholm, 1992; Lee & Söderhäll, 2002). All teleost fish appear to possess B & T-cell producing GALT (Abelli et al, 1997; Fournier-Betz et al, 2000). There is believed to be active 'dialogue' between the commensal microorganisms present in the vertebrate gut and the host mucosal immune system (Galdeano et al, 2007). Evidence of probiotic-initiated immune stimulation of GALT has been gathered in humans; including, stimulation of intestinal proinflammatory T helper 17 (T17) cells (Chow & Mazmanian, 2009) and of the secretion of the antibody, polymeric IgA, important in protecting mucosal surfaces against harmful bacterial invasion (Forchielli & Walker, 2005). Since crustaceans do not contain antigen-specific lymphocytes, or produce antibodies/immunoglobulins (Lee & Söderhäll, 2002), no beneficial stimulation is possible via such routes. Consequently, any probiotic producing such immunomodulation in a vertebrate animal will not necessarily be of any benefit to an invertebrate host. Since fish possess an adaptive, specific immune system; vaccination is a viable means of preventing outbreaks of disease. However, the greatest mortality is suffered not by adult animals but by fry and juveniles, often too small to receive such vaccines. In addition, newly hatched larval fish do not yet possess a mature, stable intestinal microflora (Verner-Jeffreys et al, 2003) or an active specific immune system (Skjermo & Vadstein, 1999). Therefore, beneficial bacteria administered via either feed or growout water could provide a viable means of preventing or minimising disease outbreaks and stock loss in such young animals (Skjermo & Vadstein, 1999).

In penaeid aquaculture, stock loss through disease is a greater problem than in finfish culture (Moriarty, 1998; Jayasree et al, 2006; Lemonnier et al, 2006; Soto-Rodríguez et al, 2006; Phouc et al, 2009). The risk of disease in shrimp farming increases with culture intensity and high stocking densities. The replacement of polyculture with monoculture also facilitates the spread of disease (Kautsky et al, 2000). For example, the rapid, high demand for (largely wild caught) *L. vannamei* broodstock and larvae in the late 1990's resulted in the global spread of Taura Syndrome virus in less than 10 years (Bonami et al, 1997; Phalitakul et al, 2006). Poor water quality and insufficient waste removal leads to overloading of metabolites and environmental degradation, putting the animals under stress and increasing their susceptibility to pathogens. Excessive fluctuations in abiotic factors such as oxygen levels, salinity and temperature, also increase stress and vulnerability to disease.

There are several potential approaches to disease control in penaeid aquaculture; these include improved animal husbandry and farm management as well as the administration of immunostimulants and probiotics (including bioremediation). Currently, however, the most widely utilised method of penaeid disease control involves the use of antimicrobials, such as antibiotics and chemotherapeutic agents (Angulo, 1999; Report of a joint FAO/OIE/WHO expert consultation on antimicrobial use in aquaculture and antimicrobial resistance, Anon 2006; Ninawe & Selvin, 2009). Improvements in animal husbandry and farm management, such as the use of non-earthen ponds, improved water filtration and aeration, can be effective in reducing the likelihood and severity of a disease outbreak. Used in isolation, however, they are insufficient to prevent stock loss should an outbreak occur. Crustaceans lack the specific immune system of vertebrates, relying on non-specific (humoral and haemocyte mediated) responses such as phagocytosis, melanisation, encapsulation

and coagulation (Smith & Chisholm, 1992; Chisholm & Smith, 1995). Haemolymph-borne plasma recognition proteins and non-self-recognition molecules are responsible for initiating and amplifying crustacean immune responses (Lee & Söderhäll, 2002). Research also indicates the presence of a family of antimicrobial peptides, named penaeidins, in *L. vannamei* (Destoumieux et al, 1997). As stated earlier in this section, invertebrates do not possess lymphoid tissue or the kind of adaptive, specific immune system observed in vertebrates. There has, however, been conjecture as to whether they possess some form of adaptive immune 'memory', prompting debate over the validity of the concept of crustacean 'vaccines'. Until lately, however, no mechanistic evidence supported such conjecture (Smith et al, 2003). Recent studies in lobsters (Mori & Stewart, 2006) and woodlice (Roth & Kurtz, 2009) have demonstrated specific enhancement of haemocyte phagocytosis following 'vaccination'. Despite this, considerable further research is required and the use of vaccines for disease control in penaeid aquaculture is currently still uncertain (Rowley & Powell, 2007). The use of immunostimulatory compounds has been suggested as having potential for disease suppression in crustaceans. In many instances, however, the lines between immunostimulant, vaccine and probiotic are blurred, as many potential immunostimulants are microbial products (Irianto & Austin, 2002; Smith et al, 2003). Lipopolysaccarides (from Gram-negative bacteria), glucan (from yeast), peptidoglycans (from lactic acid bacteria) and killed bacteria have all been evaluated as immunostimulants in Crustacea (Smith et al, 2003). Despite this, evidence for the efficacy of immunostimulation in crustaceans is at best incomplete and at worst contradictory, with research reporting potentially beneficial and detrimental effects (Smith et al, 2003; Li et al, 2009).

When used correctly, antibiotics and other chemotherapeutic agents can be highly effective in containing and treating bacterial disease outbreaks within shrimp farms. It is when they are used incorrectly, or excessively, that substantial problems arise. Overuse of antibiotics greatly increases the likelihood of creating antibiotic resistant strains of bacterial pathogens. Antibiotics commonly used in penaeid aquaculture to control infection by *Vibrio* spp., such as chloramphenicol, furazolidone, oxytetracycline and streptomycin, have all experienced substantial declines in efficacy due to irresponsible use (Farzanfar, 2006). This coupled with the time and great cost involved in developing new antibiotics, has led to a shortage of effective antimicrobials in aquaculture. Attempts within the industry are being made to reduce the prophylactic use of the remaining effective antibiotics; consequently these drugs should now only be regarded as a therapeutic and not a preventative measure. However, a survey of Thai shrimp farmers in 2000 indicated that 60% still administered antibiotics prophylactically and 20% incorrectly used them to treat viral infections (Gräslund et al, 2002). This problem is not restricted to antibiotics, chlorine, used to kill zooplankton in shrimp hatcheries prior to stocking has been found to stimulate the development of antibiotic resistance genes in bacteria (Moriarty, 1999). In addition to this, the use of antibiotics and chemotherapeutic agents, such as chlorine, does not kill all bacteria present. Subsequently, any surviving microorganisms rapidly proliferate and dominate the microflora as their competitors for nutrients, oxygen, etc have been removed (Farzanfar, 2006). Such occurrences involving the domination of the microflora by *Vibrio harveyi* have been observed frequently in Thai shrimp farms (Moriarty, 1999).

The argument for the use of probiotic bacteria in combating disease outbreaks and improving water quality in penaeid aquaculture facilities has recently gained

momentum. Despite the potential displayed by probiotics in agriculture and finfish culture, the crustacean aquaculture industry has been slow to grasp the potential of beneficial microorganisms. The majority of research carried out in the 1990's focused on the *in vitro* activity and the effects of probiotics on growth, feed utilisation and survival parameters (Maeda & Chiu-Liao, 1992; Moriarty, 1998; Rengpipat et al, 1998; Gatesoupe, 1999; Verschuere et al, 2000). Only within the last decade (predominantly the last three years) has any noteworthy research been conducted on the *in vivo* abilities and modes of action of these microorganisms in a crustacean host (see Table 4) (Irianto & Austin, 2002; Gullian et al, 2004; Ravi et al, 2007; Castex et al, 2008; Decamp et al, 2008; Kesarcodi-Watson et al, 2008). For instance, the lactic acid bacterium, *Pediococcus acidilactici*, has been shown, under field trial conditions, to reduce the occurrence of Summer syndrome (*V. nigripulchritudo* infection) (Castex et al, 2008). *L. plantarum* has been observed to induce immune modulation, improved immune ability and increased resistance to *V. alginolyticus* in *L. vannamei* (Chiu et al, 2007). *Bacillus subtilis* administration has been shown effective in protecting juvenile *L. vannamei* against vibriosis (Balcázar & Rojas-Luna, 2007). As well as bacteria administered via feed, the addition of beneficial bacterial strains to the growout water can be utilised to improve water quality and remove pathogenic bacteria (Ma et al, 2009; Wang & He, 2009). This is technically classified as bioremediation and is often a simpler (and cheaper) means of reducing the prevalence of/and susceptibility to disease causing microorganisms in shrimp aquaculture facilities. The role of the microflora of growout water in disease outbreaks should not be ignored; the exchange of water and removal of organic sludge from ponds is insufficient and must be combined with the establishment of a mature, stable, non-harmful microflora. This is especially vital in shrimp hatchery, nursery and broodstock facilities as aquatic

organisms derive their intestinal microbiota from the water they inhabit (Harris, 1993).

The majority of *L. vannamei* producing countries are developing nations for whose economies the massive revenue the penaeid culture industry generates is vital. A global estimate from one study indicated that a shrimp farm worker can earn 1.5 – 3 times as much as in other unskilled jobs (World Bank, NACA, WWF and FAO, 2002). Consequently, within the industry there is a constant demand for means by which to maximising production yields and profit while minimising stock loss. The role of probiotics within a multifaceted strategy for disease prevention/control in penaeid aquaculture is made clear by the importance of a healthy gut microbiota in crustaceans, particularly during larval and post larval developmental stages. The role of microorganisms in improving water quality and reducing aquatic pollution and farm running costs should also not be underestimated.

Table 4. Prospective probiotics evaluated for shrimp aquaculture.

Strain	Source	Evaluated for	Effective dose / Mode of application	Reference
Bacillus S11	Black tiger shrimp *Penaeus monodon*	Growth & survival of black tiger shrimp, *Penaeus monodon*.	2.5% BS11 (~1010 CFU g-1) in 3 kg of feed	Rengpipat et al. 2003
Bacillus subtilis BT23	Shrimp culture ponds	Against the growth of *Vibrio harveyi* isolated by agar antagonism assay from *Penaeus monodon*	10^6 - 10^8 CFU ml^{-1} for 6 days	Vaseeharan & Ramasamy 2003
Pseudomonas sp. PM11 & *Vibrio fluvialis* PM17	Gut of farm reared sub-adult shrimp	Immunity indicators of *Penaeus monodon*	PM11 (10^3 bacteria ml^{-1} for 3 days) - PM17 (10^3 bacteria ml^{-1} for 7 days)	Alvandi et al. 2004
Arthrobacter XE-7	Isolated from *Penaeus chinensis*	Protection of *Penaeus chinensis* post-larvae from pathogenic vibrios e.g. *V. parahaemolyticus V. anguillarum* & *V. nereis*	10^6 CFU ml^{-1}	Li et al. 2006
Bacillus subtilis & *B. megaterium*	Marine environment	Production of digestive enzymes proteases, carbohydrolases and lipases	Potential application in shrimp feeds	Solano & Soto 2006
Paenibacillus spp. *B. cereus* & *Pa. polymyxa*	Seawater, sediment and marine fish-gut samples	Activity against pathogenic *Vibrio* spp.	10^4 and 10^5 CFU ml^{-1}	Ravi et al. 2007
Lactic acid bacteria	Shrimp gut	Survival of marine shrimp *L. vannamei* challenged with *V. harveyi*	Liquid diet supplemented with B6 strain (10^8 CFU ml^{-1})	Vieira et al. 2007
Lactobacillus plantarum	Shrimp isolate	Immune response & microbiota of G.I. tract of *L. vannamei* challenged with *V. harveyi* & *V. alginolyticus*	10^{10} CFU kg^{-1} diet/ 10^8 CFU kg^{-1} feed	Chiu et al. 2007
V. alginolyticus UTM 102, *Bacillus subtilis* UTM 126, *Roseobacter gallaeciensis* SLV03, & *Pseudomonas aestumarina* SLV22	Gastrointestinal tract of adult shrimp *Litopenaeus vannamei*	Antagonistic activity against the shrimp-pathogenic bacterium, *Vibrio parahaemolyticus* PS-017	Feed supplement	Balcázar et al. 2007
Bacillus subtilis UTM 126	Shrimp pond culture	Protection against vibriosis in juvenile *Litopenaeus vannamei*	10^5 CFU g^{-1}	Balcázar & Rojas-Luna 2007
Bacillus licheniformis	Shrimp pond	Intestinal microbiota & immunity of the white shrimp *Litopenaeus vannamei*	*B. licheniformis* suspension of 10^4 CFU ml^{-1} for 40 days	Li et al. 2007
Pediococcus acidilactici	Strain MA 18/5M, CNCM	Survival of *Litopenaeus stylirostris* against Vibriosis caused by *Vibrio nigripulchritudo*	Probiotic-coated pellet feed	Castex et al. 2008
B. subtilis, *B. natto* & *B. licheniformis*	Not stated	Growth & digestive enzyme activity of *Litopenaeus vannamei*	1.5 to 7.5% supplemented to the feed	Gómez & Shen 2008

1.7 Aims of Thesis

The aims of the studies reported in this thesis are:

- The determination of whether lactobacilli can improve the health of crabs (*Carcinus maenas*).

- The *in vitro* assessment of the modes of action and efficacy of commercial terrestrial probiotics against crustacean bacterial pathogens, with emphasis on their potential applications in penaeid aquaculture.

- The isolation and identification of bacteria from healthy shrimp microbiota that may be of use as probiotics, with the possibility of future commercial application.

- The assessment of the ability of non-pathogenic members of the Vibrionaceae for growth interference/inhibition of *Vibrio* pathogens of penaeid shrimp.

Chapter 2

Investigation of the potential of the terrestrial bacterium, *Lactobacillus plantarum*, as a probiotic for marine crustaceans

Abstract

The terrestrial lactic acid bacterium, *Lactobacillus plantarum*, is utilised as a probiotic supplement in both humans and agricultural animals. However, its potential as a probiotic in marine aquaculture, specifically Crustacea, has been largely unexplored. The aim of the work was to investigate *L. plantarum*'s suitability for this role with regards to its viability within a marine crustacean host, the European shore crab, *Carcinus maenas*. In addition, any effects of *L. plantarum* administration on markers for the crustacean non-specific immune system were also assessed. The viability of the probiotic was assessed through the sampling of faeces and hepatopancreas over two feed trials. The effects on immune parameters were investigated via examination of phenoloxidase and phagocytic activity, as well as by determining total and proportional circulating haemocyte numbers. Oral administration of *L. plantarum* had no demonstrable effect on immune parameters of the shore crab *C. maenas*. In addition, the bacteria had no discernible effect on the growth or survival of the animal. The finding of greatest significance was the ability of *L. plantarum* to survive both the marine environment and transit through the gastrointestinal tract of the shore crab. However, maintenance of the probiotic required continual oral administration. In conclusion, the application of *L. plantarum* as a potential probiotic in marine crustacean aquaculture is certainly feasible; however, further investigation into whether it provides any benefit to the host organism is required.

2.1 Introduction

Decapod crustaceans make up a considerable portion of the global aquaculture market, a market which over the last few decades has become the world's fastest growing food production sector (Moriarty, 1999; Farzanfar, 2006). The global losses due to disease, in shrimp aquaculture alone, exceeded three billion US dollars in 2006 (Farzanfar, 2006). As described in Chapter 1, the prophylactic administration of strains of probiotic bacteria is now widely perceived as potentially being one of the most effective ways of preventing disease outbreaks in modern intensive aquaculture (Verschuere et al, 2000; Irianto & Austin, 2002).

Prior to the commencement of the work outlined in this study in 2006, no evidence of previous research involving lactic acid bacteria supplementation in Crustacea could be found. Species of *Carnbacterium* and *Lactobacillus*, have, however, been tested as potential probiotics in marine fish with beneficial effects observed (Jöborn et al, 1997; Robertson et al, 2000; Carnevali et al, 2004; Kim & Austin, 2006).

The selection of *Carcinus maenas*, a species of little commercial interest, as the model for this study was made primarily due to the large body of research and expertise available with regard to its non-specific immune responses (e.g. - Smith & Ratcliffe, 1980 (a, b); Schnapp et al, 1996; Hauton et al, 1997 (a,b); Johansson et al, 2000). Furthermore, a local 'crab-farm' (JW Aquaculture Research, Ltd.) was assessing the potential of *C. maenas* for the bait market and the company had a need for the development of a cheap, but effective diet based on waste fish. Initial studies had also examined the potential of dietary chitin as an immune stimulant in this

species (Powell & Rowley, 2007). JW Aquaculture Research closed this pilot 'crab-farm' in 2006-7. In addition, the abundance of experimental animals was also a significant factor in the decision, given the presence of a large, readily accessible, local population.

Consequently the focus of this study was the investigation of the potential of *Lactobacillus plantarum* as an orally administered probiotic in a model decapod crustacean, the European shore crab, *Carcinus maenas*. The primary aims were to determine whether *L. plantarum* is capable of surviving and colonising the gastrointestinal tract of *C. maenas* and whether supplementation has any affect on several markers for non-specific immunity.

2.2 Materials and methods

2.2.1 Trial 1: An initial six week *Lactobacillus plantarum* feed trial in *Carcinus maenas*

A preliminary study focused on determining the feasibility of adapting a commercially available terrestrial probiotic, *L. plantarum*, for use in marine decapod species. In this first trial, the probiotic's potential effects on basic haemolymph parameters and the bacteria's ability to survive transit through a marine decapod host, were examined.

2.2.1.1 Animals & experimental design

Crabs were collected exclusively from the Prince of Wales Dock, Swansea, via baited pots. Only adult male common shore crabs, *C. maenas*, of at least 55mm carapace width and possessing both chelipeds were selected. Control and probiotic diet groups were housed in 40L tanks, each tank containing 10 animals, within the re-circulating aquarium at Swansea University. Each group was fed 30g of formulated feed (~3g per animal), 4 times per week for a period of 42 days. Faecal material and haemolymph were sampled on a weekly basis. After 42 days, 5 animals from the probiotic group and four from the control were sacrificed for hepatopancreas sampling. Normal haemolymph sampling was suspended during week 4 in order to develop a phenoloxidase activity assay.

2.2.1.2 Preparation of feed

The feed utilised for this trial comprised a gelatine embedded, partially disassociated haddock fillet (*Melanogrammus aeglefinus*) purchased fresh from a local fishmonger, using a method modified from that of Powell & Rowley (2007). The control group received the standard feed whilst the feed administered to the probiotic group contained 1% by mass of lyophilised pure culture of *L. plantarum* supplied by Cultech Ltd. (Baglan Industrial Park, UK).

Diet production involved the skinning and the removal of any remaining bone from the haddock fillet, followed by the partial disassociation of the flesh (for ~30 sec) in a domestic food processor. In the case of the control diet, 500 g of haddock flesh was required to produce 1.214 kg of finished feed while in the case of the probiotic feed 494.64 g of flesh and 5.36 g of probiotic was required to produce an equal amount of probiotic feed. Once weighed, 714 ml of molten 5% gelatin solution (at 35°C) was added to the haddock flesh. The solution comprised 35.7g of porcine type A, 300 bloom gelatin (Sigma-Aldrich Inc. G2500-500G) dissolved in 714 ml of deionised, distilled water (ddH_20), in a water bath set at 40°C. In the case of the probiotic feed, the gelatin solution was cooled to 35°C prior to the addition of the lyophilised *L. plantarum* to maximise bacterial viability. The probiotic was mixed using a magnetic stirrer before the suspension was added to the haddock flesh. Once thoroughly mixed, the feeds were allowed to set at 4°C for a minimum of one hour before being divided into 30 g blocks. The blocks were then wrapped in aluminium foil and stored at -20°C until required. Samples of feed (2 g per week) were dispatched to Cultech Ltd. to determine the stability of the probiotic within the feed (see Appendix 2). To achieve this, the feed block was broken up and 1 g added to 90 ml of maximum recovery

diluent (MRD) (Difco™, cat# 218971). The sample was then vortexed until thoroughly disassociated, for ca. 5 min. A volume of 50 µl was then spread plated in triplicate on *Lactobacillus* deMan, Rogosa and Sharpe agar medium (MRS) (Oxoid Ltd., cat# CM0361) a medium highly specific for lactic acid bacteria. The plates were then incubated at 37°C for 48 h, followed by colony counts and confirmation of *L. plantarum* via testing with the API 50 CHL (V5.1) sugar fermentation test (BioMérieux UK Ltd., Basingstoke, UK) as per the manufacturer's instructions.

2.2.1.3 Haemocyte counts

Total haemocyte counts (THC) were performed using a Neubauer haemocytometer under a Leitz Wetzlar light microscope. Haemolymph was extracted from the same 5 individuals in each diet group on a weekly basis (individuals were identified via the application of coloured markings). After surface sterilisation with ethanol, 200 µl of haemolymph was drawn using a 1 ml syringe and 21G needle from the first joint of the fourth pereopod (walking leg), into an equal volume of ice cold marine anticoagulant (MAC) (Appendix 1; Söderhäll and Smith, 1983). Haemolymph/MAC solution (25 µl) was then pipetted onto the haemocytometer, with counts performed under x100 magnification.

Differential haemocyte counts (DHC) were performed as for THC, with 200 µl of haemolymph drawn into 200 µl of 7.4% formalin MAC (same composition as outlined previously but with the replacement of 17 ml of ddH_20 with 37% formaldehyde solution). Slides were then prepared by placing 100 µl of diluted cell suspension into a Shandon cytocentrifuge (1000 rpm; 5 min). The slides were then allowed to dry at room temperature before being fixed in absolute methanol and

stained using undiluted Wright's stain (~2 min). The numbers and proportions of each cell type were then calculated using a minimum of 200 cells/per slide. Haemocyte types were identified using the morphological criteria of Bauchau (1981).

2.2.1.4 Haemolymph protein

Serum protein concentrations were determined for the remaining 5 animals in each group using the bicinchoninic acid assay kit (Pierce and Warriner, Chester, UK) as per the manufacturer's instructions for 96 well plates. Haemolymph (500 µl) was drawn from each crab and centrifuged (2500 x g; 8 min; 4°C), with the serum then divided into 100 µl aliquots. One aliquot was then used in the determination of serum protein, while the remainder were stored at -80°C for use in any future testing. All samples were measured in triplicate using a bovine serum albumin (BSA) standard curve (100-2000 µg ml^{-1}) run on the same plate.

2.2.1.5 Persistence of *L. plantarum* in the G.I. tract of *C. maenas*

Pooled faecal samples were collected from each diet group on a weekly basis, with an approximate volume of 125 µl of faeces drawn into an equal volume of filter-sterilised (0.22 µm) aquarium water. The faecal suspension was then mixed with 250 µl of storage medium (50% glycerol, 3% sodium chloride solution) and stored at -20°C until the end of the trial.

Samples of hepatopancreas were also taken aseptically at the end of the trial (requiring the sacrifice of 5 animals from the probiotic group and 4 from the control) the samples were stored at -20°C, in an equal volume of the storage medium.

Post trial work on the recovery of viable *L. plantarum* from the faecal and hepatopancreas samples was conducted at Cultech Ltd. (project's industrial sponsor). After manual homogenisation of each sample for 30 sec, seven x10 dilutions were made. After the initial round of plating, the number of dilutions required was lowered to three. All samples were plated in duplicate, on to five growth media; MRS agar for *Lactobacillus*, MRS agar (plus 1% sodium chloride), MRS agar (plus 3% sodium chloride), nutrient agar and sheep blood agar. Each dilution was pipetted, 5 x 10µl equally spaced drops, onto a quarter plate. The drops were allowed to dry at room temperature (18°C) before the plates were incubated anaerobically at 30°C for four days. After four days, a colony count and preliminary colony identification was performed. Those colonies identified as possible *L. plantarum* then underwent testing with the API 50 CHL (V5.1) sugar fermentation test (BioMérieux UK Ltd., Basingstoke, UK) as per the manufacturer's instructions.

Those colonies positively identified as *L. plantarum* by the API 50 CH test, were streaked and isolated before being store in agar slopes/stabs. This 'recovered' strain was then used as the stock supply of *L. plantarum* for salinity tolerance comparison trials.

2.2.1.6 Salinity tolerance of *L. plantarum*

The salinity tolerance trials required the culturing of both the original lyophilised *L. plantarum* used in the production of the crab feed and the *L. plantarum* recovered from the crab faeces under saline conditions using a method modified from that of Vásquez et al (2003). Adapted incubations of both 'types' of *L. plantarum* were

composed of suspensions of live bacteria in 10 ml of filter sterilised sea water. The concentration of live bacteria (CFUs) in the lyophilised *L. plantarum* incubation was 1.58×10^{10} L^{-1}. To minimise sources of error, the concentration of CFUs in the 'recovered' *L. plantarum* incubation was adjusted to 1.58×10^{10} L^{-1}. Initial time 0 samples of the 'recovered' strain were drop plated in order to determine accurately the number of viable CFUs present at the start of the incubation. The suspensions were then incubated at 18°C.

Both incubations were sampled every 6 h over the course of 24 h (i.e. at 6, 12, 18 and 24 h respectively). Sampling involved the removal of 100 µl of the incubation (after gentle shaking) prior to dilution with filter sterilised sea water. Eight dilutions were made with each subsequent dilution equating to one tenth the concentration of the previous, with the exception of the first. Each dilution was then drop plated in duplicate onto MRS agar containing 1% sodium chloride. The plates were incubated anaerobically at 28°C for 72 h. The *L. plantarum* colonies of the dilution showing an optimum growth pattern were counted and the CFUs present in the incubation at that time were calculated.

2.2.1.7 Statistical analysis

To evaluate differences in cell counts and haemolymph protein concentrations between diet groups an ANOVA together with a Bonferroni multiple comparisons post test was used. This followed the determination of normal distribution of the data via the application of a Kolmogorov-Smirnov test. All values are shown as arithmetic means ± 1 standard error of the mean (S.E.M).

2.2.2 Trial 2: An 11 week *Lactobacillus plantarum* feed trial in *Carcinus maenas*

A second, longer term feed trial focused on determining any potential effect of *L. plantarum* administration on two *C. maenas* immune parameters (phagocytosis and phenoloxidase activity). In addition, the ability of *L. plantarum* to persist in the gastrointestinal tract of crabs after the cessation of probiotic feed was also investigated.

2.2.2.1 Animals & experimental design

Animal selection criteria, husbandry and feeding schedule remained unaltered from the initial trial (Trial 1). Each diet group again consisted of 10 animals, individually identifiable by applied coloured markings, with each group again divided into two subgroups of five individuals. Sampling was conducted every two weeks (with the exception of week 11), in an attempt to minimise mortality due to stress. One subgroup was utilised for faecal sampling, the other for haemolymph samples utilised to assess phagocytic and phenoloxidase activity. In addition, probiotic administration was halted in week 7 of the trial in order to ascertain the length of time *L. plantarum* was able to persist in the decapod gastrointestinal tract after the cessation of probiotic feed. In week 11 (end of trial) three animals from each diet group were sacrificed, with hepatopancreas samples taken to attempt recovery of *L. plantarum.* The materials and methods utilised for the preparation of feed and for faecal/hepatopancreas microbiology remained unaltered from the previous trial.

2.2.2.2 Haemocyte phagocytic activity

The phagocytic activity of haemocytes was determined utilising stained yeast zymosan as described in detail previously (Mayrand et al, 2005). Diagnostic slides (8x8mm wells) were first washed in MilliQ water, followed by immersion in an ethanol bath (70%) for 1 h. They were again rinsed in MilliQ water before being heat sterilised at 200°C to remove any bacterial contamination.

A suspension of type A yeast zymosan in marine saline (Appendix 1) was stained in a 5% neutral red solution and the concentration adjusted to ca. 5 x 10^7 zymosan ml^{-1}.

After surface sterilisation with alcohol, 200 µl of haemolymph was drawn, using a 1 ml syringe and 21G needle, from the first joint of the fourth pereopod (walking leg) into 800µl of ice cold marine anticoagulant (MAC). The suspension was then centrifuged (10,000 x g; 1 min; 4°C) before re-suspending the haemocytes in ice-cold marine saline. The THC was enumerated using a Neubauer haemocytometer and cell concentration adjusted to ca. 5 x 10^6 cells ml^{-1}. Haemocyte suspension (50 µl) was pipetted into each well of a diagnostic slide, with two replicates performed for each sample. The slides were left for 10 min at RT to allow cell adhesion, before gentle rinsing with ice-cold marine saline to remove any unattached haemocytes. Once prepared, 25 µl of the yeast zymosan suspension was pipetted into each well and the slide placed on a filter paper soaked in marine saline (in a 90 mm Petri dish) before being incubated at 18°C in darkness for 1 h. Post incubation, any non-engulfed zymosan were washed off with ice-cold marine saline and the slides were then fixed for 10 min in 5% formalin before being rinsed with ddH_2O and allowed to dry.

To determine the phagocytic activity, the first 100 haemocytes encountered under the x100 oil immersion objective were examined for the presence of engulfed yeast

zymosan. If engulfed zymosan accounted for less than 10% of cytoplasm volume (equating to 0-1 zymosan) then the cell was regarded as being 'inactive'. Phagocytic activity was expressed as a percentage (i.e. % phagocytic haemocytes).

2.2.2.3 Phenoloxidase activity

The phenoloxidase assay used was modified from that reported by Smith & Söderhäll (1991). All glassware and unsterilised plasticware utilised was rendered pyrogen and endotoxin free by immersion in a 0.1% E-Toxa-Clean solution (Sigma 210-3) for a minimum of 2 h, followed by washing in MilliQ ddH_2O. In addition, glassware was then heat sterilised at 200°C for 2-4 h.

After surface sterilisation with alcohol, 300µl of haemolymph was drawn, using a 1 ml syringe and 21G needle, from the first joint of the fourth pereopod (walking leg), into equal volume of ice cold MAC. The number of samples required the staggering of incubations to minimise the effects of premature activation of the phenoloxidase within the haemolymph. Each incubation contained the same number of control and probiotic diet group samples, to prevent the introduction of additional variation.

The contents of the syringe were then carefully transferred to a 1.5 ml Eppendorf tube containing an additional 300 µl of ice cold MAC. The suspension was then centrifuged, (800 x g; 5 min; 4°C). The cells were then washed twice in cacodylate sucrose buffer (CSB) (Appendix 1) with care being taken not to resuspend the pellet. After the second wash the supernatant was drawn off and discarded and the pellet resuspended in 500 µl of sodium cacodylate buffer (Appendix 1). The sample was then manually homogenised on ice for 30 sec before centrifuging (16,000 x g; 20 min; 4°C). The supernatant, referred to as the haemocyte lysate supernatant (HLS) was

aliquoted, 50 µl in triplicate, into a 96 well, flat-bottomed plates (Nunc® Sterile, prod# TKT-180-070U). Control blanks were also included where the HLS was replaced with sodium cacodylate buffer & ddH_2O. Fifty microlitres of 0.1% trypsin solution was then added to each well and the plate incubated at 20°C for 30 min. During this period, the concentration of protein within the remaining HLS was determined using a bicinchoninic acid assay kit (Pierce and Warriner, Chester, UK) as per the manufacturer's instructions, with a BSA standard (100-2000 µg ml^{-1} range). After 30 min, the plate was removed and 50 µl of 0.3% L-Dopa solution (Appendix 1) was added to each well before incubating for a further 20 min at 20 °C. Absorbance at 492 nm was then recorded using a microplate reader (Multiscan Ascent, Dynex Labsystems®, Middlesex, UK).

Phenoloxidase specific activities were calculated using the formula outlined in Appendix 1 with results expressed as ΔOD 492 nm/min/mg protein.

2.2.2.4 Statistical analysis

Total haemocyte counts were only performed in the course of conducting the phagocytic activity assay, therefore, no statistical analysis of these data was conducted. To evaluate any differences in phagocytic and phenoloxidase activity between the diet groups, an ANOVA together with a Bonferroni multiple comparisons post test was used. This followed the determination of normal distribution of the data via the application of a Kolmogorov-Smirnov test. Values were shown as arithmetic means ± 1 standard error of the mean (S.E.M).

2.3 Results

2.3.1 Trial 1: An initial six week *L. plantarum* feed trial in *C. maenas*

2.3.1.1 Probiotic stability in feed

L. plantarum levels within the gelatinous diet stored at -20°C exhibited a gradual decline in the number of viable lactobacteria. The CFUs declined from 8.25 x 10^7 g^{-1} to 5.00 x 10^7 g^{-1} during the three week period of sampling (Figure 1).

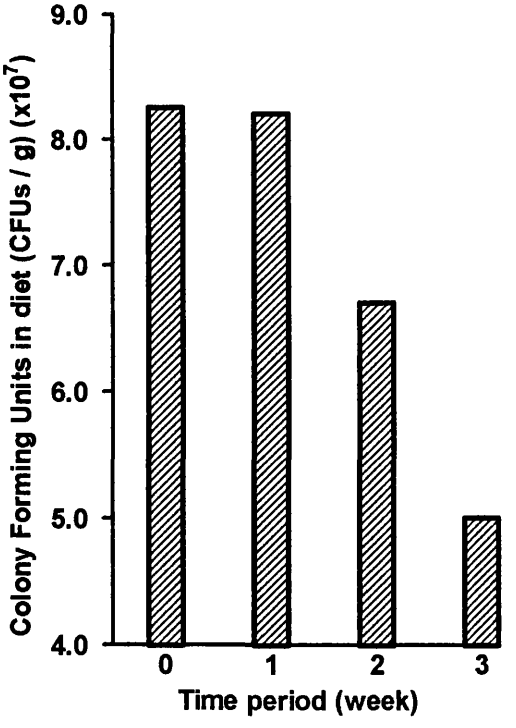


Figure 1. Number of *L. plantarum* colony forming units recovered from experimental diet (single estimation performed)

2.3.1.2 Haemocyte counts

The results obtained in this trial are shown in Figures 2-5. Both total (THC) haemocyte numbers and the proportions (DHC) of the three cell types displayed a high degree of variability throughout the trial period. There was, however, no significant difference between the two diet groups with regard to either THC or DHC at any time point during the 6 week period (ANOVA with Bonferroni multiple comparison post test, $P<0.05$) (Figures 2-5).

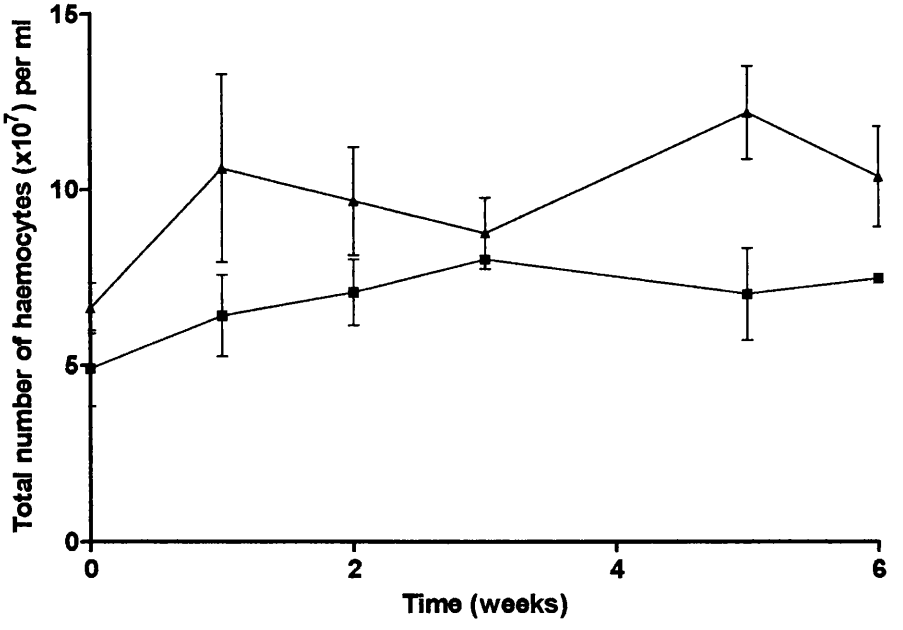


Figure 2. Total haemocyte count for control (---) and probiotic (---) diet groups over 6 week feeding period. Mean values ± SEM, n=5

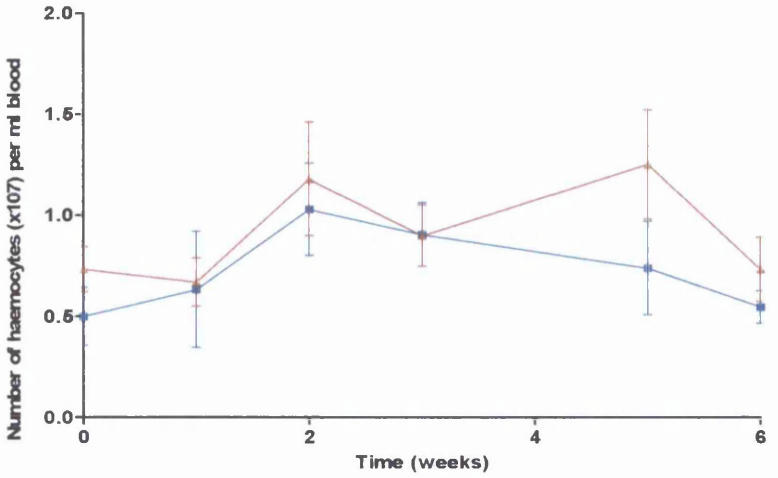


Figure 3. Number of granulocyte-type haemocytes observed in haemolymph samples from control (---) and probiotic (---) crabs over the 6 week feeding period. Mean values ± SEM, n=5

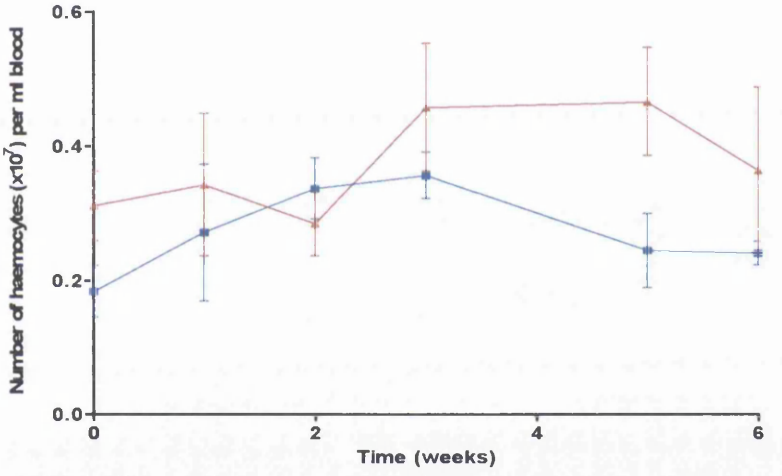


Figure 4. Number of hyaline-type haemocytes observed in haemolymph samples from control (---) and probiotic (---) grps over the 6 week feeding period. Mean values ± SEM, n=5

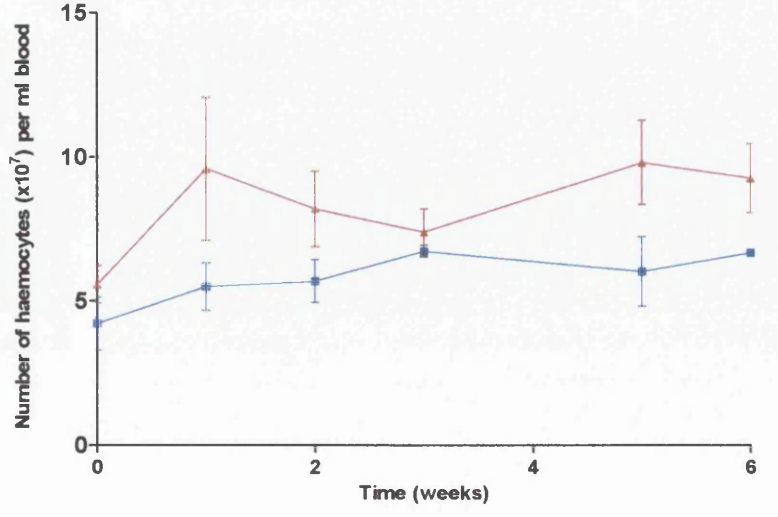


Figure 5. Number of semigranulocyte-type haemocytes observed in haemolymph samples from control (---) and probiotic (---) grps over the 6 week feeding period. Mean values ± SEM, n=5

2.3.1.3 Total haemolymph protein

The total serum protein concentrations of crabs on the control and probiotic diets were similar over the duration of the trial (Fig. 6). No significant difference in serum protein concentration was observed between the control and probiotic diet groups, with the exception of at week 2 (ANOVA with Bonferroni multiple comparisons post test; $P<0.05$).

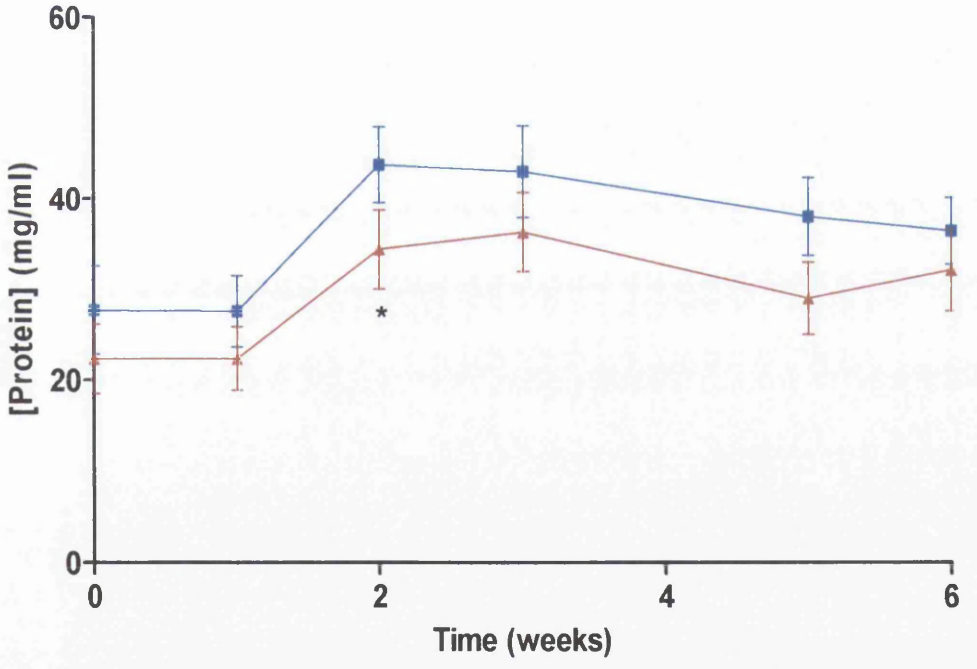


Figure 6. Serum [protein] (mg/ml) observed in haemolymph samples from control (---) and probiotic (---) crabs over the 6 week feeding period. Mean values ± SEM, n=5, * $P<0.05$ compared with control

2.3.1.4 Persistence of *L. plantarum* in the GI tract of *C. maenas*

As shown in Table 1, *L. plantarum* was found in faecal samples as the trial progressed, with CFUs increasing from 0 at week 0 (basal sample) to $>1.60 \times 10^7$ g^{-1} faeces at week 6. Trace levels (ca. 10^2. g^{-1} wet weight) of *L. plantarum* were also identified in two of the control group faecal samples at weeks 1 & 5. *L. plantarum* was absent from the remaining control group samples.

Although *L. plantarum* was not observed in the majority of the control faecal samples, a second bacterial type on MRS was prevalent, characterised by small, round, translucent colonies. This bacterium was not further identified as it was clearly (through colony morphology and Gram staining) not a species of *Lactobacillus*. The hepatopancreas contained very little culturable material with very low concentrations of the probiotic bacteria recovered in samples from two probiotic group animals, yielding 0.96×10^3 and 0.40×10^2 CFUs g^{-1} of hepatopancreas (Table 2). *L. plantarum* was not observed in any control group hepatopancreas samples (Table 2).

Table 1. *L. plantarum* colony forming units present in pooled faecal samples gathered on a weekly basis.

Sample period (weeks)	Number of *L. plantarum* CFUs recovered (CFU per g faeces wet weight)	
	Control diet group	Probiotic diet group
0	0	0
1	2.4×10^2	0
2	0	5.4×10^6
3	0	4.5×10^6
4	0	4.9×10^4
5	8.0×10^2	$>1.6 \times 10^7$
6	0	$>1.6 \times 10^7$

Table 2. *L. plantarum* colony forming units present in hepatopancreas.

Number of *L.plantarum* CFUs recovered (CFU per g hepatopancreas wet weight)	
Control diet group	Probiotic diet group
0	0
0	1.0×10^3
0	0
0	0.4×10^2
0	0

2.3.1.5 Salinity tolerance of *L. plantarum*

Incubations comparing the haline tolerances of original lyophilised cultures of *L. plantarum* with those recovered from the faeces of probiotic-fed crabs (pure cultures positively identified via API 50 CHL testing as *L. plantarum*) yielded interesting results. Figure 7 displays the mean CFUs derived from four replicate incubations of both lyophilised and recovered faecal *L. plantarum*. The most significant feature was the apparent large drop in viability in the lyophilised population observed in the initial 6 h. After this point, the lyophilized and faecal incubations showed no significant variation in survival/mortality. Colony forming units within the recovered, faecal incubations exhibited no significant variations over the 24 h incubation period (ANOVA with Bonferroni multiple comparisons post test; $P<0.05$) with CFUs ml^{-1} varying from $1.95x10^7$ at 0 h, to $1.31x10^7$ at 24 h.

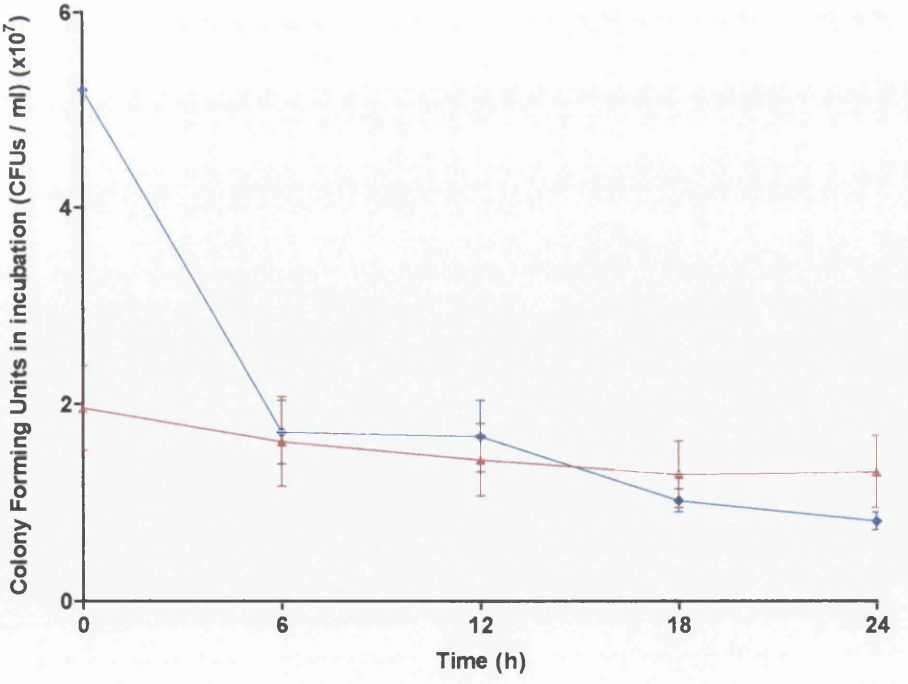


Figure 7. Survival of original lyophilised *Lactobacillus plantarum* (---) administered in feed compared to that recovered from crab faeces (---). Mean values ± SEM, n=4.

2.3.2.1 Trial 2: Phagocytic activity against zymosan

During Trial 2 mortality within the probiotic group was high, 70% over the 11 weeks compared to only 20% among the control crabs. The cause of this mortality was an as yet unclassified rickettsia-like bacterial pathogen endemic in the local wild *C. maenas* population during late summer (Eddy et al, 2007).

To ascertain whether *L. plantarum* administration affected the phagocytic activity of haemocytes in *C. maenas*, a phagocytic activity assay was performed using zymosan as a test particle. There was no evidence that feeding of *L. plantarum* had any effect on the ability of haemocytes to phagocytose zymosan (Figure 8). No significant differences were observed between the control and experimental groups at any point during the trial, nor was there any difference between the basal (week 1) and final probiotic samples (ANOVA with Bonferroni multiple comparisons post test; $P<0.05$).

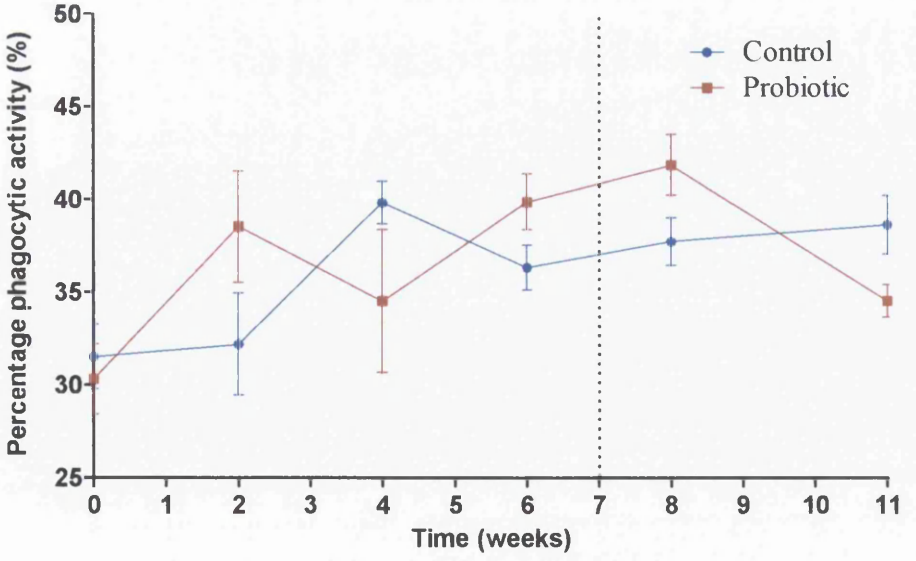


Figure 8. Percentage phagocytic activity of *C. maenas* haemocytes over 11 week trial (probiotic supplement ceased in week 7). Mean values ± SEM, n=5.

2.3.2.2 Phenoloxidase activity

An assessment of the phenoloxidase activity of haemocyte lysate supernatant was performed as a further indicator of a possible change in immune status following probiotic treatment.

There was a high degree of variation in the phenoloxidase activity within each diet group over the trial period (Figure 9). However, as was the case with phagocytic activity, no significant variation between the control and probiotic groups was observed over the 11 week period (ANOVA with Bonferroni multiple comparisons post test; $P<0.05$).

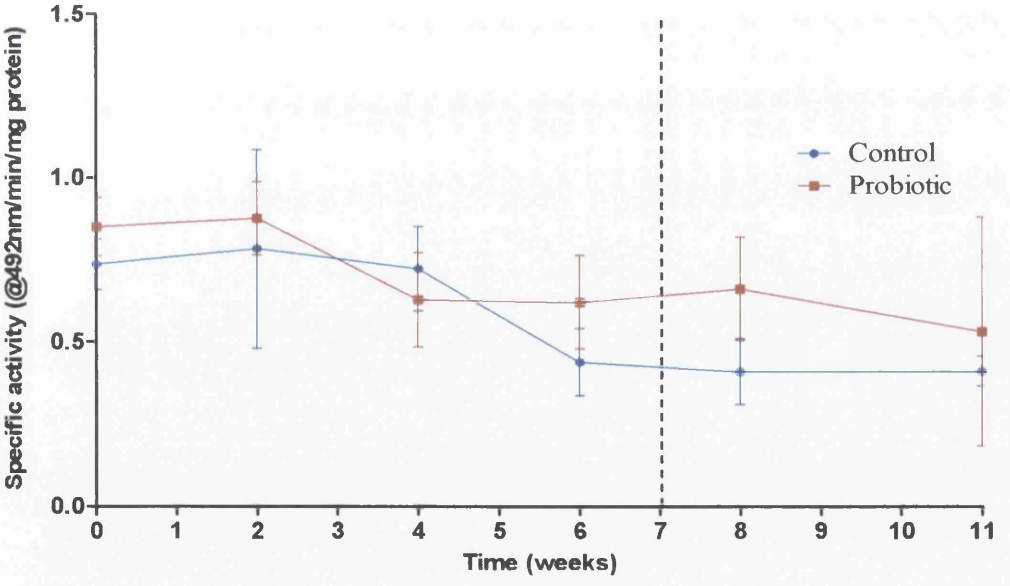


Figure 9. Specific activity of phenoloxidase in haemocyte lysate supernatant obtained from *C. maenas* haemolymph over 11 week trial (probiotic supplement ceased in week 7). Mean values ± SEM, n=2-5.

2.3.2.3 Persistence of *L. plantarum* in the GI tract of *C. maenas*

As in the initial trial, the work to recover and isolate viable probiotic from stored faecal material collected during this second trial was conducted at Cultech Ltd. (Table 3). *L. plantarum* accumulated in the faeces of those crabs fed probiotic during the period of its administration (up to week 7) increasing from zero CFUs at week 0 to 1.9×10^6 CFU g^{-1} by week 6 (Table 3). Levels of *L. plantarum* in the faecal samples declined rapidly during the week after the switch to the non-probiotic diet, disappearing entirely by week 9 (Table 3). *L. plantarum* was identified in the week 4 control group faecal sample, but was absent from the remaining samples.

As in the initial trial, hepatopancreas samples contained little culturable material and low concentrations of the probiotic were recovered from two of the probiotic-fed animals, yielding mean values of 0.6×10^3 and 0.1×10^3 CFU g^{-1} wet weight (Table 4). *L. plantarum* was not recovered from any control group hepatopancreas samples.

Table 3. *L. plantarum* colony forming units present in pooled faecal material.

Sample period (weeks)	Number of *L. plantarum* CFUs recovered (CFU per g faeces wet weight)	
	Control diet group	Probiotic diet group
0	0	0
2	0	1.1×10^3
4	1.3×10^5	4.3×10^4
6	0	1.9×10^6
8	0	2.1×10^4
9	0	0
10	0	80
11	0	0

Table 4. *L. plantarum* colony forming units present in hepatopancreas at week 11.

Number of *L. plantarum* CFUs recovered (CFU per g hepatopancreas wet weight)	
Control diet group	Probiotic diet group
0	0
0	0.6×10^3
0	0.1×10^3

2.4 Discussion

The majority of previous work involving the administration of potential probiotic organisms to marine crustacean species has focused on their gross effects with regards to growth and mortality (Rengpipat et al, 1998; Gomez-Gil et al, 2000; Rengpipat et al, 2000; Gullian et al, 2004; Balcázar et al, 2007; Wang, 2007). By comparison, until recently there has been relatively little investigation of the potential effects of probiotic bacteria supplementation may have on the immune parameters of cultured marine invertebrates.

This current study described in this section was primarily aimed at determining the potential of utilising a terrestrial probiotic, in this case *Lactobacillus plantarum*, for use in a marine decapod crustacean species. The selection of *L. plantarum* as the primary test probiotic was the result of several factors. Firstly, lactic acid bacteria including *L. plantarum*, have been used extensively and successfully as terrestrial probiotics for many years (e.g. Ringø & Gatesoupe, 1998; Cebeci & Gürakan, 2003; Coeuret et al, 2004). *L. plantarum* has been approved for use as a human probiotic within the European Union (Directive; 70/524/EEC, JLO 297:15/11/2001); (Coeuret et al, 2004) and is used commercially by the project's industrial partner (Obsidian/Cultech Ltd., Baglan) in a number of proprietary supplements supplied to major companies in the U.K. Consequently, a continuous supply of homogeneous, lyophilised *L. plantarum* was available throughout the study. *L. plantarum* is regarded as a hardy microbe, capable of surviving transit through the stomach of terrestrial vertebrates (de Vries et al, 2006) whose potential as an aquatic/marine probiotic has been outlined previously (Gildberg et al, 1995, Carnevali et al, 2004; Vázquez et al, 2005; Chiu et al, 2007). In addition, lactobacilli are principally non-pathogenic (in

mammals at least) and have been found naturally in the alimentary canal of many fish species (Ringø et al, 1995; Ringø & Gatesoupe, 1998; Ringø et al, 2000; Robertson et al, 2000).

In the current study, the effect of *L. plantarum* administration on four basic immune parameters was assessed alongside its potential to colonise the G.I. tract of crabs. No significant variation in total haemocyte populations, or the individual blood cell types between the diet groups of the initial trial was observed. Therefore, it is possible to regard the administration of *L. plantarum* over a 6 week period as having no discernible effect on the circulating haemocyte levels of healthy crabs. Total and differential haemocyte counts are sometimes considered as indicators of possible immune activity (Tsing et al, 1989; Johansson et al, 2000). A decrease in circulating haemocyte numbers is often observed in the initial stages of an infection as those haemocytes responsible for the production of antimicrobial peptides and cell-mediated responses migrate out of the haemolymph into the affected tissues (Schnapp et al, 1996; Bachére et al, 2004). If the animal survives the initial post-infection period (ca. 48 h), there often follows a proliferation of haemocytes as production is stimulated returning the circulating numbers to pre-infection level (Bachére et al, 2004). Therefore, a relative decrease in the granular-type (antimicrobial peptide expressing) cells within the haemolymph might indicate migration of these cells into the tissues and the instigation of an immune-stimulatory effect (Bachére et al, 2004).

Over the trial period both groups displayed a near identical pattern of haemolymph protein concentration, with no significant divergences observed. The sizable increase in the concentration of haemolymph serum protein observed in both diet groups

during the second week of the trial is likely to be due to the animals' improved diet, as it persisted for the remainder of the feeding period.

L. plantarum supplementation elicited no change in either the phagocytic or phenoloxidase activities of circulating haemocytes. Both of these parameters are well established markers for non-specific immunity in invertebrates (Brookman et al, 1989; Rowley et al, 1990; Smith & Söderhäll, 1991; Dyrynda et al, 1995; Hauton et al, 1997 (a,b); Cerenius et al, 2008; Li et al, 2008). The high mortality suffered by the probiotic diet group during week 8, however, makes these results potentially unreliable due to the reduction in sample size. This mortality suffered by the probiotic diet group crabs during the second trial can be attributed to an outbreak of 'milky disease'. This is a systemic infection often observed in the local *C. maenas* population during the summer, whose causative agent is believed to be an as yet unidentified rickettsial-like α-proteobacterium (Eddy et al, 2007).

The recovery of large quantities of *L. plantarum* from faeces of the probiotic fed animals in both trials indicates that *L. plantarum* survives transit through the crustacean gastrointestinal tract. In addition to this, the number of CFUs was shown to increase as the trial progressed, provided that bacterial supplementation was not halted. This seems to indicate that while *L. plantarum* did not actively colonise the digestive tract, as defined in Fuller (1992) it did appear capable of not only maintaining a stable transitory population but also proliferating whilst in transit. The inability of lactobacilli to colonise the GI tract is not unusual when administered to healthy animals (Robertson et al, 2000). However, the low levels of probiotic detected in two of the three hepatopancreas samples taken 4 weeks after the cessation of supplementation, may give an indication that low level, longer term colonisation of

the hepatopancreas may be possible. *L. plantarum* was detected in control group faecal samples on three occasions over the two trials. Given the sporadic nature of its appearance and extremely low levels in comparison to the equivalent probiotic samples, the source of the bacteria should be regarded as being due to contamination. This is further supported in that *L. plantarum* is not a natural component of the marine microflora and has never been isolated from the GI tract of *C. maenas*.

Previous studies have shown that some lactobacilli are capable of producing osmoregulatory proteins in order to better survive in environments with high dissolved solute concentrations (Piuri et al, 2003). The contribution of the proteolytic system peptide supply is thought to be significant in the process of osmotic adaptation in these bacteria (Piuri et al, 2003; Wood et al, 2001). In the current study *L. plantarum* appeared to survive well in sea water suggesting it may have some potential as a probiotic for marine invertebrates and vertebrates. Although no significant adaptation to saline conditions was observed, the fact that *L. plantarum* exhibited tolerance to haline conditions (Wood et al, 2001) and survived transit through a marine decapod host, were important findings and indicate the probiotic's suitability for further testing.

Temperature may be a more important parameter than salinity with regard to survival and activity of lactobacilli (Vázquez et al, 2003). Anecdotal observation indicates that *L. plantarum* typically grows best aerobically at 30°C. Therefore, *L. plantarum* may exhibit improved survival and potential probiotic activity in a tropical decapod species such as the Pacific white shrimp, *Litopenaeus vannamei* where water temperatures are somewhat higher.

In conclusion, the current study has shown that oral application of the probiotic, *L. plantarum* has no demonstrable effects on a range of immune parameters in shore

crab, *C. maenas*. Furthermore, there were no obvious effects of the probiotic on the growth and survival of these crabs. Of interest was the finding that this terrestrial microbe survives well in sea water and transit through the GI tract of crabs, but requires continual administration to maintain any presence in the gut.

Chapter 3

Selection and *in vitro* screening of an array of micro-organisms for expression of antagonistic activity against a panel of potential pathogens of shrimp

Abstract

This aspect of the project comprised two components; initially, the provision of a range of bacterial isolates (both identified and unidentified strains) in pure culture for the *in vitro* assessment of their potential as crustacean probiotics. Second to this was the development and optimisation of an array of screening assays designed to identify any anti-microbial abilities of these isolates toward a selection of potential crustacean bacterial pathogens. Bacterial strains were gathered from three distinct sources, (i) commercially utilised terrestrial probiotics (3 species of lactic acid bacteria), (ii) isolates from the microflora of cultured Pacific white shrimp, *Litopenaeus vannamei* (114 isolates) and (iii) *Vibrio* spp. with no record of pathogenicity toward marine organisms (seven *Vibrio* spp. and 4 shell disease isolates). From these sources, five potential probiotic strains displaying anti-pathogen activity were identified using four principal screening techniques (a fifth; cross-streaking, was used initially and discarded as ineffective). The five selected potential crustacean probiotics were; *Lactobacillus plantarum*, *Pediococcus acidilactici* (NCIMB 8018), a suspected strain of *Carnobacterium maltaromaticum* (isolated from *L. vannamei*), *Vibrio alginolyticus* (NCIMB 1339) and *Vibrio gazogenes* (NCIMB 2250).

3.1 Introduction

Lactic acid bacteria have been tested and utilised as probiotic feed supplements in humans, agricultural animals and teleost fish for many years (e.g. Gatesoupe, 1991; Garcia-de-la-Banda et al, 1992; Gildberg et al, 1997; Cebeci & Gürakan, 2003; Carnevali et al, 2004; Coeuret et al, 2004; de Vries et al, 2006). In addition, both *Lactobacillus plantarum* and *Pediococcus acidilactici* are already licensed for use in animal feedstuffs within the European Union (European Commission, 2004 - Community Register of Feed Additives; pursuant to regulation (EC) No 1831/2003 Rev. 44). *P. acidilactici* is also "generally recognised as safe" (G.R.A.S.) by the United States Food & Drug Administration (Salminen et al, 1998). These organisms had, at the time of screening, never been evaluated with regard to their potential as probiotics for cultured marine crustaceans.

The significance of searching for probiotic micro-organisms within the naturally occurring microbial flora of the host species (in this case *L. vannamei*) is that any organism isolated would already be suited for survival and colonisation of the host, in particular the early life stages where it would be of most benefit (Gatesoupe, 1991; Verschuere et al, 2000). In the case of shrimp, several probiotics have been found following this approach, including *Vibrio alginolyticus*, *Vibrio* spp., *Pseudomonas* spp., *Bacillus* spp. S11 and *Thalassobacter utilis*, (Gatesoupe, 1999; Irianto & Austin, 2002; Farzanfar, 2006).

Investigation of the Vibrionaceae as a source of potential probiotics also shows promise as previous studies have reported probiotic (anti-microbial) activity of such bacteria (Prasad et al, 2005; Fjellheim et al, 2007).

Studies outlined in this chapter sought to find a range of potential probiotic bacteria for shrimp (*L. vannamei*) culture. They also assessed the strengths and weaknesses of a range of *in vitro* screening assays for antagonistic activity of such bacteria against known bacterial pathogens of shrimp.

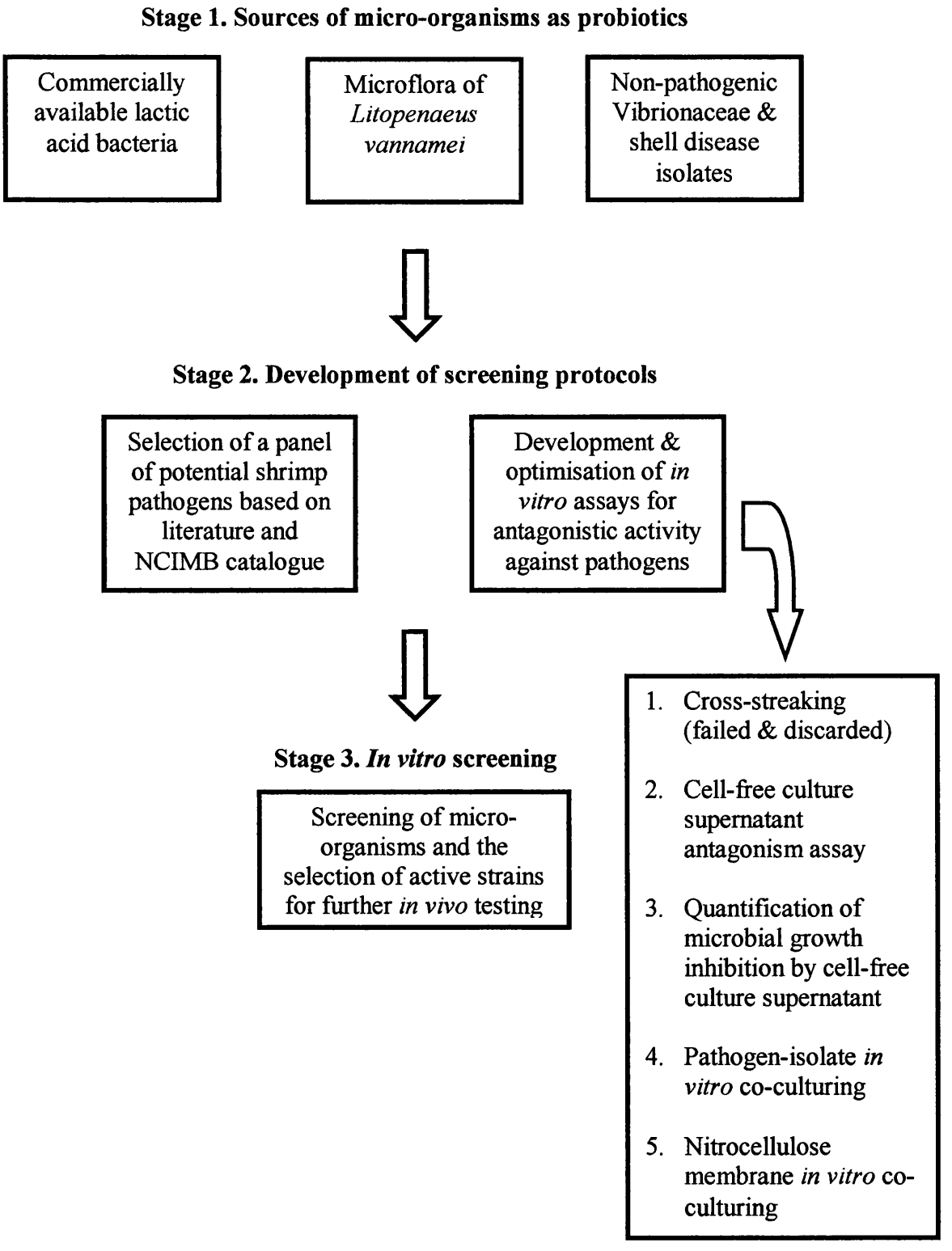
Thus, the specific aims of this chapter were:

- The isolation of bacterial strains from the microflora of healthy post-larval and juvenile Pacific white shrimp, *L. vannamei*, under varying growth conditions and media (Figure 1).

- The development and optimisation of a battery of *in vitro* screening assays to elucidate any potential, novel crustacean probiotics (Figure 1) with particular relevance to the direct growth interference/inhibition by either live culture or extracellular products.

- The *in vitro* screening of isolated strains, along with species of lactic acid bacteria and selected *Vibrio* spp.

Figure 1. Approaches taken in the isolation and screening of bacterial strains during the search for potential crustacean probiotics.



3.2 Materials and Methods

3.2.1 Sourcing of potential probiotics for Pacific white shrimp, *Litopenaeus vannamei*

3.2.1.a Commercially available lactic acid bacteria

The three species of lactic acid bacteria selected for screening for anti-*Vibrio* activity were *Lactobacillus plantarum*, *Pediococcus acidilactici* (NCIMB 8018) and *Lactobacillus curvatus* subsp. *curvatus* (NCIMB 9716). *L. plantarum* was screened due to its extensive use as a terrestrial probiotic supplement in numerous commercial products, as well as by the project's industrial sponsor, Cultech/Obsidian Ltd. Consequently, large homogeneous quantities of the bacterium were available for experimental work and feed preparation. *L. plantarum* had also been isolated from the gastrointestinal tracts of healthy Arctic Charr (*Salvelinus alpinus*) indicating its potential as a marine probiotic (Ringø & Gatesoupe, 1998). The selection of *P. acidilactici* and *L. curvatus* subsp. *curvatus* was based on their prior history as terrestrial probiotics and the availability of pure cultures from NCIMB Ltd. (Aberdeen, UK). In addition, viable cells of *P. acidilactici* are the principal component of Bactocell PA™ a probiotic feed supplement for fish (source; European Food Safety Authority).

L. plantarum was obtained in lyophilised form from Cultech Ltd. Baglan, UK, (stored at 4°C) and grown aerobically on MRS media at 30°C, when required. *P. acidilactici* and *L. curvatus* subsp. *curvatus* were maintained on slopes of MRS media and also stored at 4°C. The techniques described in Sections 3.2.2.a and 3.2.2.b were utilised to

determine whether any of the three strains possessed an ability to interfere or inhibit the growth of potential shrimp pathogens. The remaining assays outlined in Section 3.2.2 could not be utilised for the above bacteria as the type of media required for pathogen culture was unsuitable for the growth of lactic acid bacteria.

3.2.1.b Natural microflora of healthy cultured Pacific white shrimp, *Litopenaeus vannamei*

The general microflora of healthy post-larvae and gastrointestinal tract microflora of adult, Pacific white shrimp, *Litopenaeus vannamei*, were screened for potential novel probiotic bacterial strains.

Six post-larval *L. vannamei* of two size classes (weighing 0.5 ± 0.1 g and 8 ± 0.5 g, respectively) were sampled. Animals were obtained from the CSAR facility at Swansea University. In the case of the smaller animals, a homogenate of whole shrimp was prepared (after washing with sterile 3% NaCl solution) as their size made removing intact hind gut and hepatopancreas impossible. The 8 g animals were aseptically dissected with samples of whole hind gut, including faeces, and hepatopancreas taken. All animals were euthanised on ice, prior to sampling. In addition to the animal samples, biofilm swabs from the CSAR shrimp rearing tanks were also tested. Samples were placed in 1.5 ml plastic Eppendorf tubes (sterile) containing 500 µl of sterile 3% NaCl solution before being manually homogenised (30 sec). Multiple 10 fold dilutions of the homogenates were performed (up to x 10,000) followed by spread plating, in triplicate, on the following media; TSA (Bacto™, cat#236920) (plus 2% NaCl) Marine agar (2216) (Difco™ cat# 212185) Marine agar (2216) plus 10 µg ml^{-1}, 2,4-diamino-6,7-diisopropyl pteridine phosphate

salt (Sigma-Aldrich Ltd., Dorset, U.K. Cat#D0781) Marine agar (2216) plus 100 μg ml^{-1}, 2,4-diamino-6,7-diisopropyl pteridine phosphate salt, and MRS agar (plus 2% NaCl). TSA and Marine agar plates were incubated aerobically at 25°C and checked for colony growth every 12 h. The MRS plates were incubated under aerobic and anaerobic conditions at 37°C for 72 h (optimum conditions for lactic acid bacteria growth). All visually-distinct colonies were streaked onto fresh plates. During early screenings the Gram type and gross colony morphology of isolates was recorded, according to Colomé et al (1986) (Appendix 3). All isolated strains, *ca.* a total of 114 were maintained on slopes of appropriate media and stored at 4°C.

The techniques outlined in Section 3.2.2 were then utilised to screen the isolates for indications of antagonistic activity against a panel of shrimp bacterial pathogens. A cross-streaking method was initially attempted to check for competitive interference between isolates and potential pathogens. This assay involved aseptically streaking first the potential shrimp pathogen and then the test isolate onto TSA (plus 2% NaCl) or marine agar plates before incubating at 25°C for 24 h. At the end of the incubation period the plate was inspected for any indication of pathogen growth interference. This technique was extremely unreliable and was discarded in favour of the pathogen-isolate *in vitro* co-culture method described in Section 3.2.2.b. As a consequence detailed methodology of this assay was not included in Section 3.2.2.

Partial identification of Gram positive isolates obtained from MRS agar plates displaying anti-*Vibrio* activity was made using the API 50 CHL (V5.1) sugar fermentation test (BioMérieux UK Ltd., Basingstoke, UK) as per the manufacturer's instructions.

3.2.1.c The selection of non-pathogenic *Vibrio* species as potential probiotics

The *Vibrio* spp. tested were selected from those available from the NCIMB catalogue (NCIMB Ltd, Aberdeen, UK). A literature review was undertaken, with each species assessed with regard to its suitability as a potential crustacean probiotic. Factors used to select suitable species were; evidence of prior testing as a potential probiotic and/or no previous indications of pathogenicity. Particular attention was paid to those species originally isolated from the GI tract of marine hosts. Species with any record of pathogenicity toward marine animals (vertebrate or invertebrate) were immediately discounted. The species selected for *in vitro* testing were; *Vibrio gazogenes* (NCIMB 2250) *Vibrio mediterranei* (NCIMB 13228) *Vibrio natriegens* (NCIMB 2273) *Vibrio orientalis* (NCIMB 2195) *Vibrio proteolyticus* (NCIMB 1326) *Vibrio scophthalmi* (NCIMB 13623) and *Vibrio tubiashii* (NCIMB 1336); based on information contained in Cerdà-Cuéllar et al (1997), de Schrijver & Ollevier (2000), Huys et al (2001), Oxley et al (2002) and Jawahar Abraham & Palaniappan (2004).

All seven micro-organisms were maintained on TSA (plus 2% NaCl) slopes stored at 4°C. Standard Vibrionaceae incubation conditions of 25°C for 24 h were used during culturing. The seven selected *Vibrio* spp. (plus the pathogens *V. harveyi*, *V. alginolyticus* and *L. anguillarum*) were screened using the methods outlined in Sections 3.2.2.a and 3.2.2.b. Those displaying inhibitory activity toward the initial screening panel of *V. harveyi*, *V. alginolyticus* and *L. anguillarum* were then tested against the following five crustacean pathogens, *V. campbellii*, *V. nigripulchritudo*, *V. penaeicida* and the Class 2 human and crustacean associated pathogens, *Vibrio parahaemolyticus* (NCIMB 1164) and *Vibrio vulnificus* (NCIMB 2046).

Those species which interfered with the growth of >50% of the pathogen panel were subjected to additional testing to investigate the nature of their inhibitory activity. Given that many *Vibrio* spp. exhibit pathogenicity toward crustaceans, plus the ability of bacteria to mutate/exchange plasmids, these were tested to determine whether their anti-*Vibrio* activity could be retained without the need to administer the live organism in feed. In addition to the potential probiotic-derived cell free culture supernatant, the activity of French pressed (lysed) broth culture was also assessed via the method described in Section 3.2.2.b. The French pressed culture material was prepared using a French® Pressure cell press and French® pressure cell ($^3/_8$" piston) (Sim-Aminco Spectronic Instruments Inc., Rochester, NY, USA). A cell pressure of 18,000 psi was required to successfully lyse the *Vibrio* spp. tested.

3.2.1.d Miscellaneous screened strains

In addition to the *Vibrio* spp., four unidentified bacterial strains isolated from the carapace lesions of edible crabs (*Cancer pagurus*) affected by shell disease were also screened. These strains were obtained during previous research projects and bear the prefix 'SDI'. All shell disease isolates were maintained on TSA (plus 2% NaCl) slopes stored at 4°C and cultured at 25°C (over 24 h). All four were tested alongside the *Vibrio* spp. listed in Section 3.2.1.c.

3.2.1.e Selection of a screening panel of putative bacterial pathogens of shrimp

Screening pathogens were selected based on prior evidence of pathogenicity against crustaceans, particularly shrimp. Those selected were *V. harveyi* (NCIMB 1280) *V.*

alginolyticus (NCIMB 1339), *L. anguillarum* (NCIMB 829), *Vibrio campbellii* (NCIMB 1894), *Vibrio nigripulchritudo* (NCIMB 1904) and *Vibrio penaeicida* (NCIMB 13386) (Hauton et al, 1997; Goarant et al, 1999; Liu et al, 2004; Gauger et al, 2006; Goarant et al, 2006; Lemonnier et al, 2006; Soto-Rodríguez et al, 2006). In addition to these, two class 2 crustacean associated pathogens were also selected for limited screening. These were *Vibrio parahaemolyticus* (NCIMB 1164) and *Vibrio vulnificus* (NCIMB 2046) (Sudheesh & Xu, 2001). Given that class 2 organisms are regarded as pathogenic to humans, these micro-organisms were only used to screen those isolates that had shown antagonistic potential against the initial six species. Although *V. vulnificus* is not directly pathogenic to shrimp, it is an opportunistic pathogen of humans causing primary bacteraemia, gastrointestinal illness and infection of soft tissue, either through consumption of contaminated seafood (particularly invertebrates) or via open wounds (Chiang & Chuang, 2003). Consequently, a potential crustacean probiotic displaying antagonistic activity against this microbe would be extremely desirable. All pathogen cultures were obtained from NCIMB Ltd., Aberdeen, UK, and were maintained on TSA (plus 2% NaCl) slopes at 4°C. The class 2 organisms were stored separately in a secure refrigerator.

3.2.2 *In vitro* screening processes applied to potential probiotics for evidence of antagonistic activity against a panel of putative shrimp bacterial pathogens

3.2.2.a Cell free culture supernatant antagonism assay

This technique, modified from that of Gram et al (1999), was utilised to determine whether the cell free culture supernatant of potential probiotics exhibited antagonistic

activity toward pathogenic *Vibrio* species.

Tryptic soy agar plates, TSA plus 2% NaCl and tryptic soy broth, TSB with 2% NaCl were used to culture the pathogens. Cell free culture supernatant obtained from incubation of the potential probiotic isolates was tested for antagonistic activity against selected potential pathogens. The strains of lactic acid bacteria were cultured in MRS broth (Oxoid™, cat#CM0359) at 30°C for 24 h, while those strains grown on TSA and Marine agar were incubated in TSB with 2% NaCl and Marine broth, respectively, at 25°C for the same period. At the end of the 24 h incubation period the broth cultures were centrifuged (6000 x g, 10 min at 25°C) and filter sterilisation (0.22 µm filter) in order to render them cell free (confirmed via spread plating and microscopy). Supernatants were stored at 4°C for no more 24 h prior to assay commencement.

The potentially pathogenic *Vibrio harveyi* (NCIMB 1280) and *Listonella* (*Vibrio*) *anguillarum* (NCIMB 829) were cultured in TSB (plus 2% NaCl) at 25°C for 18 h. The cell concentration of each incubation was adjusted to *ca.* 2×10^9 total bacteria ml^{-1}. Pathogen culture (100 µl) was then aseptically spread onto each plate. After 20 min (sufficient time for bacterial adhesion) 12 equidistant, 4 mm diameter wells were punched into the agar using a sterile cork borer. Forty microlitres of the probiotic cell-free culture supernatant was then added to each well with the three lactic acid bacteria represented in triplicate on each plate (i.e. 3 x 3 wells). A negative control of the appropriate uninoculated broth was added to the remaining three wells. Each plate was run in duplicate. Plates were then incubated at 25°C and inspected for evidence of growth inhibition/interference at 24 and 48 h.

In cases where pathogen growth interference was clearly observed, further trials were conducted to ascertain whether varying the incubation period and temperature of the

growth of the test probiotic impacted on activity. The effects of varying the incubation period were assessed by utilising cell-free supernatant produced from culture samples extracted daily over a 7 day incubation. The activity of cell-free culture supernatant (24 h) was assessed after exposure to temperatures of 65°C and 100°C. In addition, the supernatants were also subjected to several cycles of freeze-thawing at -80°C.

All isolates displaying antagonistic activity against members of the pathogen screening panel were examined in more detail via the method described in Section 3.2.2.d

3.2.2.b Pathogen-isolate *in vitro* co-culturing

Modified from Pilet et al (1995), this technique was utilised to rapidly determine the *in vitro* competitive or inhibitory activity of a potential probiotic against a series of potential bacterial crustacean pathogens.

Twenty five millilitres of *V. harveyi* culture, previously grown up in TSB (plus 2% NaCl) for 18 h at 25°C, was aseptically mixed with 475 ml of molten TSA (plus 2% NaCl) at 40°C. Sufficient TSA powder and NaCl were used for a 500 ml final volume. The agar-pathogen suspension was aseptically dispensed into twenty 90 mm Petri dishes. This process was repeated for *V. alginolyticus* and *L. anguillarum*, with 20 plates sufficient to test 20 isolates in duplicate. The remaining three potential *Vibrio* pathogens and the class 2 organisms listed in Section 3.2.1.e were only utilised for the screening of the non-pathogenic *Vibrio* spp. and shell disease isolates.

The potential probiotic strains were grown under the same culture conditions as the pathogens (TSB plus 2% NaCl; 18 h at 25°C). Fifty microlitres of isolate broth culture

was pipetted onto the surface of the pathogen inoculated plate (once set) with care taken to maintain the broth as a single drop. Four, 50 µl samples were equidistantly positioned on each plate (Petri dish marked prior to addition). Two isolates were screened per plate with each plate run in duplicate. Post inoculation, the plates were left face up for 30 min at RT to allow for bacterial adhesion. The plates were incubated at 25°C and checked for evidence of growth interference/inhibition at 18, 48 & 72 h (or 24 & 96 h in the case of the non-pathogenic *Vibrio* spp. and shell disease isolates).

Any isolates displaying antagonistic activity against members of the pathogen screening panel were examined in more detail via the method described in Section 3.2.2.d.

3.2.2.c Nitrocellulose membrane *in vitro* co-culturing

The technique utilised was modified from a method described by Sambrook & Russell (2001). Hind guts (including faeces) and hepatopancreas were aseptically dissected from 2 adult Pacific white shrimp, *L. vannamei* (8 ± 0.5 g), obtained from the CSAR facility at Swansea University. Each sample was placed in 500 µl of sterile 3% NaCl solution and manually homogenised for 30 sec. Dilutions of x 1000 and x 10000 were made, with 100 µl of each dilution spread, in duplicate, onto moistened sterile, 90 mm nitrocellulose membranes (Millipore™ (UK) Ltd., Watford, UK; RAWP09025). The membranes were then carefully placed onto TSA (plus 2% NaCl) plates, with care taken to ensure no air was trapped between the membrane and agar. The plates were incubated at 25°C for 24 h.

V. harveyi, *V. alginolyticus* and *L. anguillarum* impregnated TSA (plus 2% NaCl) plates were produced prior to the end of the incubation period, via the method outlined in Section 3.2.2.2.

Post incubation, the inoculated membranes were aseptically removed from the plates and placed on sterile, 90 mm filter papers (moistened with sterile 3% NaCl solution) with the contact surface facing upward. The plate surface was then photographed as a record of colony distribution (e.g. Figure 7; Section 3.3.2). A fresh sterile nitrocellulose membrane was placed onto the inoculated 'master' membrane (ensuring no trapped air between the membranes) followed by a second moisten 90 mm filter paper. The orientation of the membranes with regard to the original plate were marked before a 90 mm Petri dish was then pressed down (gently) onto the stack of membranes/papers, with care taken to ensure evenly distributed pressure. The filter papers were removed and the membranes were then carefully peeled apart using sterile blunt forceps. The upper 'replica' membrane was placed onto one of the *V. harveyi* impregnated plates with care taken to avoid smearing. The aforementioned was then repeated for the *V. alginolyticus* and *L. anguillarum* impregnated plates and for each subsequent 'master' membrane.

The plates were incubated at 25°C and checked at 8 h intervals over 24 h for colony formation and evidence of interference of pathogen growth. Colonies indicated as potential inhibitors of the pathogens were isolated from the original 'master' plates and stored on slopes at 4°C, for further conformation of activity via methods described in Sections 3.2.2.b and 3.2.2.d The 'master' membranes were stored at -20°C on sealed TSA plates plus 2% NaCl and 10% glycerol.

3.2.2.d Quantification of microbial growth inhibition by cell free culture supernatant

A positive result obtained in any of the assays outlined in Sections 3.2.2.a-c indicated the potential of the test bacterium to interfere with/inhibit the growth of potentially pathogenic *Vibrio* species. This ability was quantified via the use of a more sensitive and reproducible microplate reader-based assay.

The crustacean pathogens utilised in this were *Vibrio harveyi* (NCIMB 1280) and *Vibrio alginolyticus* (NCIMB 1339). Both of these species are considered pathogenic for a range of crustaceans (Karunasagar et al, 1994; Gomez-Gil et al, 2004; Liu & Chen, 2004; Lio-Po et al, 2005; Jayasree et al, 2006). *V. alginolyticus* was selected over *L. anguillarum* for its higher viability and rate of proliferation. The pathogens were cultured in TSB (plus 2% NaCl) at 25°C for 12 h prior to the commencement of the assay. Cultures (10 ml aliquots) were centrifuged (1000 x g, 5 min at 25°C) with the pellet retained and re-suspended in 5 ml of sterile 3% NaCl solution; this washing process was repeated twice. After the final wash, the pellet was re-suspended in 1 ml of 3% NaCl solution. The total cell concentration was then adjusted to *ca.* 1 x 10^9 cells ml^{-1}.

Cell free culture supernatants of the probiotic isolates were prepared via the method outlined in Section 3.2.2.a. Those supernatants obtained from lactic acid bacteria cultures were determined as having a pH of *ca.* pH 4 (Corning, Inc. ®, Model 10 pH meter, New York, USA). Aliquots of these supernatants were adjusted to pH 6.2 (that of uninoculated MRS broth) using 1M & 6M solutions of sodium hydroxide and were tested alongside the original supernatants. Consequently, the potential role of acid pH

in the anti-*Vibrio* activity of these lactic acid bacteria was also investigated during this assay.

Fifty microlitres of pathogen suspension was incubated with 100 µl of cell-free culture supernatant at 25°C with shaking for 30 min, in flat-bottomed, 96-well plates (Nunc® Sterile, prod# TKT-180-070U). All combinations of cell free culture supernatant and pathogen were included and run in triplicate on each plate, alongside positive and negative controls. For the positive control, the cell free culture supernatants were replaced by sterile 3% NaCl solution, while in the negative controls, the pathogen suspensions were replaced by sterile 3% NaCl solution. During the 30 min incubation a second 96-well plate was prepared, with corresponding wells flooded with 200 µl of sterile TSB (plus 2% NaCl). Post incubation, 50 µl from each well of the first plate (incubation) was transferred aseptically to the 3 corresponding wells of the second plate. The optical density of the second plate at 550 nm was recorded at 60 min intervals over a 24 h period (at 25°C) via a microplate reader (Multiscan Ascent, Dynex Labsystems®, Middlesex, UK).

Any isolate whose cell free culture supernatant showed antagonistic activity against *V. harveyi* and *V. alginolyticus* via this method was selected for further (*in vivo*) pathogenicity check testing as described in Chapter 4.

3.2.3 Statistical analysis

To determine any significant differences in optical density and consequently pathogen growth profiles in experimental and control incubations, an ANOVA together with a Bonferroni multiple comparisons post test was used. This followed the determination

of normal distribution of the data via the application of a Kolmogorov-Smirnov test.

All values are shown as arithmetic means ± 1 standard error of the mean (S.E.M).

3.3. Results

3.3.1 *In vitro* antagonistic activity of selected lactic acid bacteria against *Vibrio harveyi, Vibrio alginolyticus* and *Listonella anguillarum*

3.3.1.1 Cell free culture supernatant antagonism assay

The cell-free culture supernatants of *L. plantarum* and *P. acidilactici* completely inhibited the growth of *V. harveyi* and *L. anguillarum* (Table 1) producing 1.5 - 3mm diameter zones free of growth around every well (Figure 2A). The supernatant of *L. curvatus* subsp. *curvatus* produced only weak, intermittent interference of *L. anguillarum* growth, over 48 h. Consequently, it was decided not to undertake further testing of *L. curvatus* subsp. *curvatus*.

Table 1. Antagonistic activity of lactic acid bacteria cell free culture supernatant against *Vibrio harveyi* and *Listonella anguillarum*; no interference/inhibition (-), indication of interference/inhibition (+).

	Crustacean pathogen			
	Vibrio harveyi		*Listonella anguillarum*	
	24 h	48h	24 h	48h
Lactobacillus plantarum	+	+	+	+
Pediococcus acidilactici	+	+	+	+
Lactobacillus curvatus subsp. *curvatus*	-	-	-	+

The ability of *L. plantarum* and *P. acidilactici* cell-free culture supernatants to inhibit the growth of *V. harveyi* and *L. anguillarum* was undiminished by heating or freeze-thawing (Figure 2B; Tables 2 and 3). Regions of pathogen growth inhibition around wells containing the heat treated and frozen supernatants were consistent with those around wells containing untreated supernatant (Figure 2B).

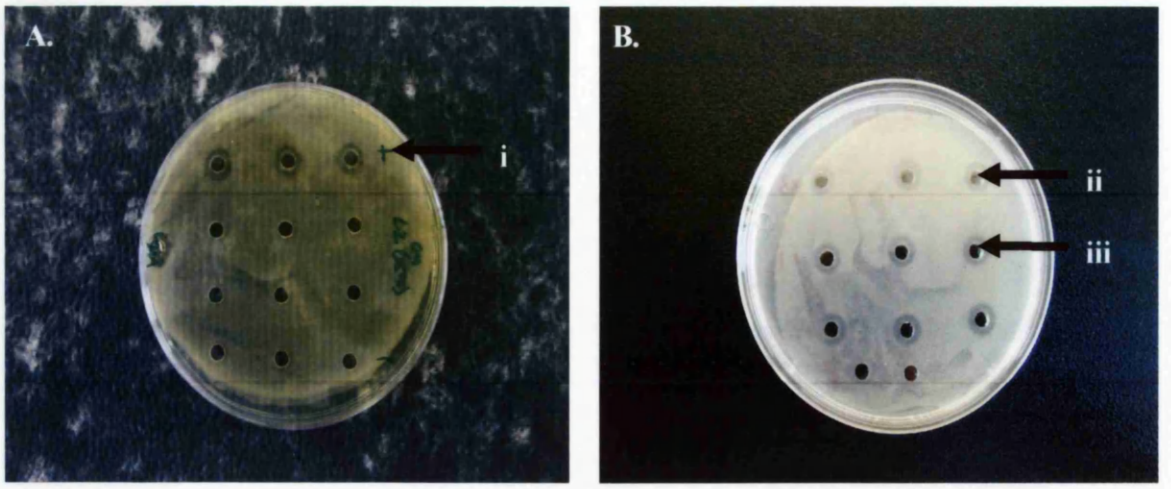


Figure 2. (**A**) Cell free culture supernatant antagonistic activity assay against *V. harveyi*. Positive result observed using cell free supernatant from *L. plantarum* (i). Supernatant in the remaining wells (from isolate 'L2') displayed no antagonistic activity. (**B**) Cell free culture supernatant antagonistic activity assay against *V. alginolyticus*. Positive results observed using cell free supernatant from *L. plantarum* subjected to freezing/thawing and heating (ii & iii).

No alteration in the antagonistic activity of the cell free culture supernatant of *L. plantarum* or *P. acidilactici* was observed with variation of culture age. Supernatants collected over the 7 days produced zones of pathogen growth inhibition comparable with the 24 h samples, i.e. zones of inhibition of 1 - 2 mm radius. Minor differences in these zones resulted from variation in agar distribution and therefore plate thickness.

Table 2. Antagonistic activity of lactic acid bacteria cell free culture supernatant against *Vibrio harveyi* and *Listonella anguillarum*, after heat exposure; no interference/inhibition (-), indication of interference/inhibition (+).

		Bacterium	
	Temperature (°C)	*Vibrio harveyi*	*Listonella anguillarum*
Lactobacillus plantarum	65	+	+
Lactobacillus plantarum	100	+	+
Pediococcus acidilactici	65	+	+
Pediococcus acidilactici	100	+	+

Table 3. Antagonistic activity of lactic acid bacteria cell free culture supernatant against *Vibrio harveyi* and *Listonella anguillarum*, after freeze-thaw cycles; no interference/inhibition (-), indication of interference/inhibition (+).

	Number of freeze-thaw cycles	Bacterium	
		Vibrio harveyi	*Listonella anguillarum*
Lactobacillus plantarum	1	+	+
Lactobacillus plantarum	3	+	+
Pediococcus acidilactici	1	+	+
Pediococcus acidilactici	3	+	+

3.3.1.2 Quantification of microbial growth inhibition by cell free culture supernatants of selected lactic acid bacteria against *Vibrio harveyi* and *Vibrio alginolyticus*

As initially indicated by the results shown in Section 3.3.1.1, cell free culture supernatant obtained from *L. plantarum* and *P. acidilactici* inhibited the growth of both *V. harveyi* and *V. alginolyticus* over 24 h, under optimum *Vibrio* growth conditions (Figures 3-6). No detectable bacterial growth was observed in any of the wells containing unaltered cell free culture supernatant at pH 4. Growth did, however, occur in wells containing the pH adjusted (pH 6.2) cell free supernatant of both lactic acid bacteria. This growth was, however, less than that observed in the appropriate positive controls (pathogen plus MRS broth; pH 6.2). In the case of pH adjusted *L. plantarum* culture supernatant, *V. harveyi* displayed a slight, but not statistically significant decline in growth/cell number after 15 h, when compared with the positive (bacteria-only) control. The effects were more pronounced for *V. alginolyticus*, where growth was statistically significantly lower over the incubation period in wells containing the pH adjusted cell free supernatant (pH 6.2) in comparison with that in the bacteria-only control (Figure 4).

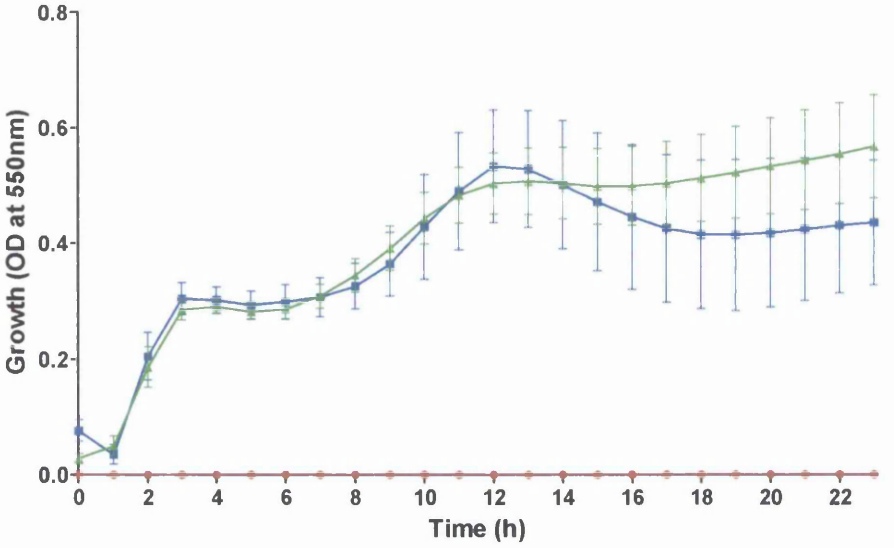


Figure 3. Growth profile of *Vibrio harveyi* in the presence of culture supernatant from *Lactobacillus plantarum*. *V. harveyi* and cell free supernatant of *L. plantarum* at pH 4 (---), *V. harveyi* and cell free supernatant of *L. plantarum* at pH 6.2 (---), *V. harveyi* and MRS broth only (---). Mean ± S.E.M, n=5.

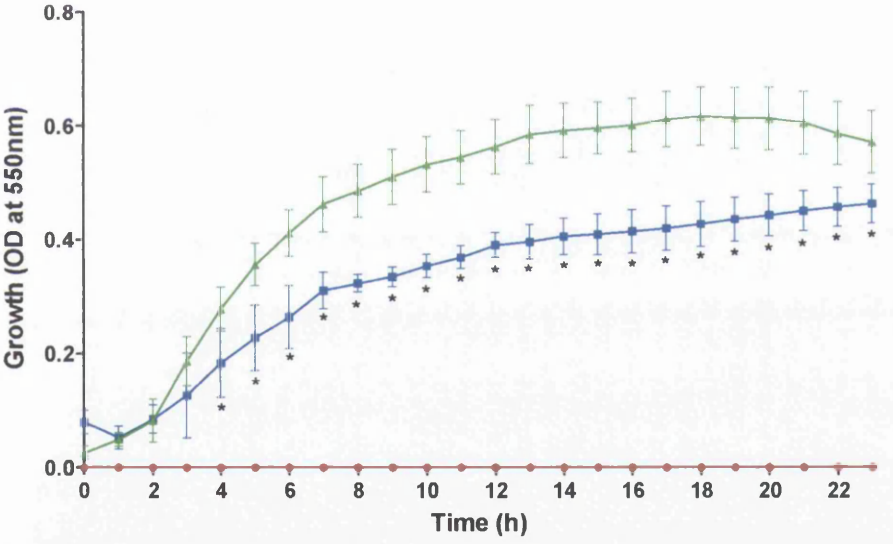


Figure 4. Growth profile of *Vibrio alginolyticus* in the presence of culture supernatant from *Lactobacillus plantarum*. *V. alginolyticus* and cell free supernatant of *L. plantarum* at pH 4 (---), *V. alginolyticus* and cell free supernatant of *L. plantarum* at pH 6.2 (---), *V. alginolyticus* and MRS broth only (---). Mean ± S.E.M, n=5, *P<0.05 compared to *V. alginolyticus* and cell free supernatant of *L. plantarum* at pH 6.2.

The pH adjusted cell free culture supernatants of *P. acidilactici* and *L. plantarum* appear equally ineffective in inhibiting the growth of *V. harveyi* (Figures 3 & 5). However, both were far more effective in their inhibition of *V. alginolyticus* producing statistically significant growth inhibition (Figures 4 & 6). The antagonistic

capacities of the cell free culture supernatants of these two bacteria appeared very similar.

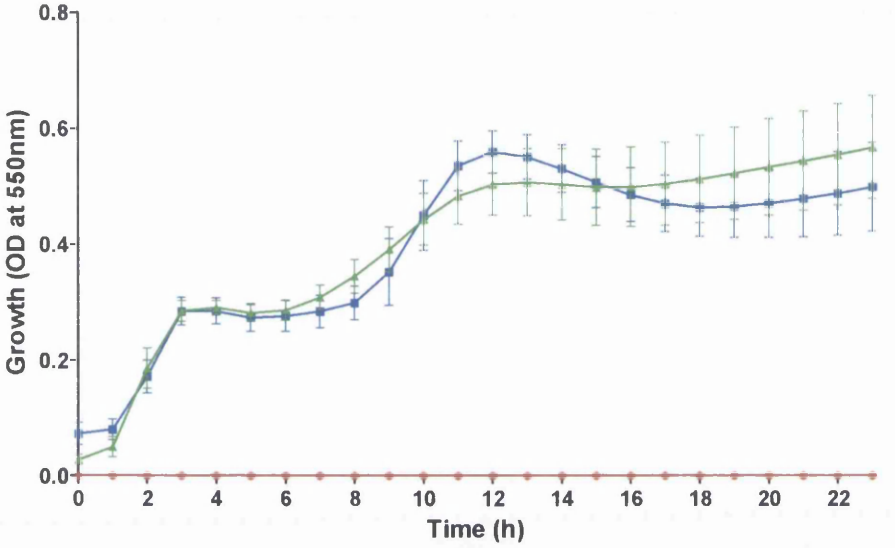


Figure 5. Growth profile of *Vibrio harveyi* in the presence of culture supernatant from *Pediococcus acidilactici*. *V. harveyi* and cell free supernatant of *P. acidilactici* at pH 4 (---), *V. harveyi* and cell free supernatant of *P. acidilactici* at pH 6.2 (---), *V. harveyi* and MRS broth only (---). Mean ± S.E.M, n=5.

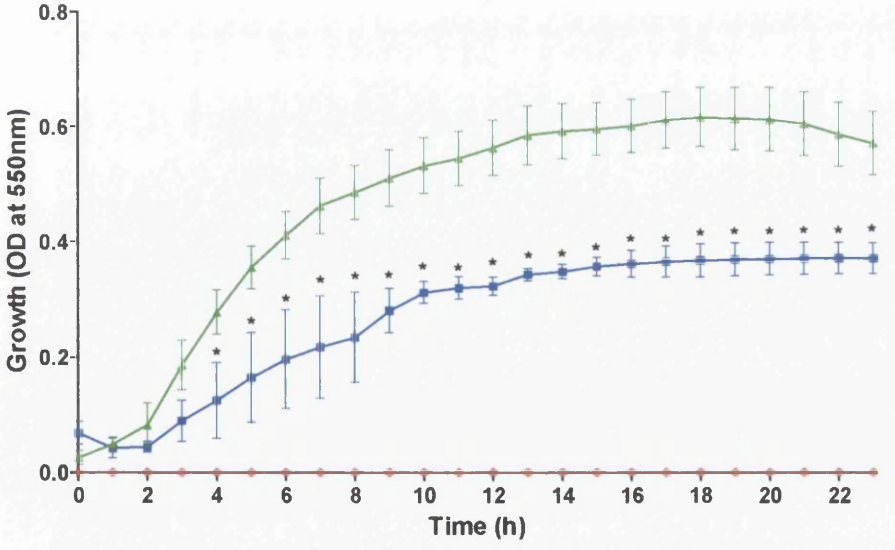


Figure 6. Growth profile of *Vibrio alginolyticus* in the presence of culture supernatant from *Pediococcus acidilactici*. *V. alginolyticus* and cell free supernatant of *P. acidilactici* at pH 4 (---), *V. alginolyticus* and cell free supernatant of *P. acidilactici* at pH 6.2 (---), *V. alginolyticus* and MRS broth only (---). Mean ± S.E.M, n=5, *P<0.05 compared to *V. alginolyticus* and cell free supernatant of *L. plantarum* at pH 6.2.

3.3.2 Screening of the microflora of the Pacific white shrimp, *Litopenaeus vannamei* for potential novel probiotics

Despite 172 potentially different bacterial strains being isolated initially (Table 4) only 114 of these were recoverable prior to screening. The *in vitro* screening of these 114 isolates sourced from *L. vannamei* suggested only four isolates that exhibited potential antagonistic activity toward the selected target *Vibrio* pathogens (Table 5). In particular, the anaerobic bacteria isolated on MRS media failed to grow after exposure to aerobic conditions, i.e. were obligate anaerobes. All methods described in Section 3.2.2 were utilised, a cross-streaking co-culture assay was attempted as an initial screen, but proved ineffective and was discarded in favour of the pathogen-isolate *in vitro* co-culturing assay (Section 3.2.2.b). Isolates 'G-B' and 'HP-B1' were isolated on MRS agar (plus 2% NaCl) and screened using the cell free culture supernatant antagonism assay (Table 5). Isolates 'G1-I3' and 'G1-I7' were initially isolated using the nitrocellulose membrane *in vitro* co-culture assay (Figure 7).

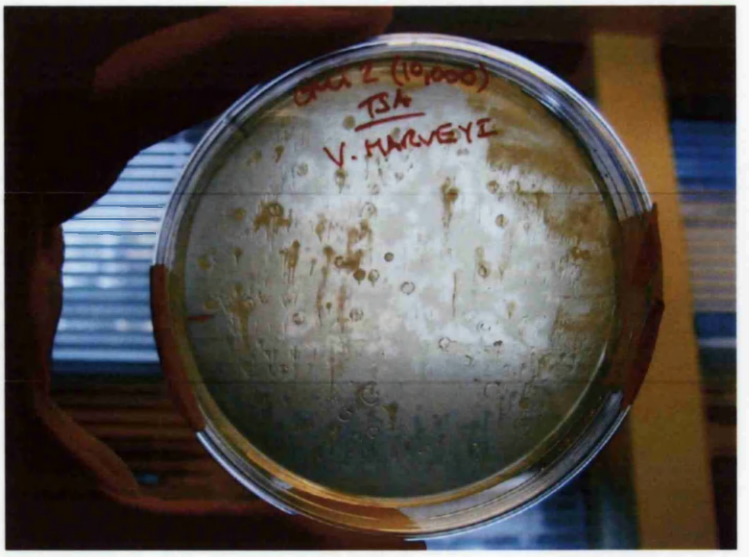


Figure 7. Nitrocellulose membrane *in vitro* co-culture (against *V. harveyi*) shown after removal of 'replica' membrane, areas of bacterial isolate growth along with regions of apparent interference/inhibition of *V. harveyi* growth are visible.

Table 4. Summary of bacterial isolates derived from the Pacific white shrimp *Litopenaeus vannamei*, including culture tank biofilm (see also Appendix 3).

Source animal	Number of isolates				
	Marine agar (Aerobic)	Marine agar (plus Vibriostatic agent) (Aerobic)	TSA (plus 2% NaCl) (Aerobic)	MRS (plus 2% NaCl) (Aerobic)	MRS (plus 2% NaCl) (Anaerobic)
Post-larvae	6	2	20	0	6
Juvenile	41	11	50	17	14
Tank biofilm	ND*	ND*	ND*	1	4

ND* = not determined.

Table 5. Bacterial isolates derived from the Pacific white shrimp *Litopenaeus vannamei*, which displayed potential antagonistic activity against shrimp pathogens; no interference/inhibition (-), indication of interference/inhibition (+), potential growth interference (P).

	Antagonistic activity against pathogens					
	Cell free supernatant			Pathogen-isolate *in vitro* co-culturing		
	V. harveyi	*L. anguillarum*	*V. alginolyticus*	*V. harveyi*	*L. anguillarum*	*V. alginolyticus*
Carnobacterium maltaromaticum (G-B)	+	-	+		Incompatible media	
Carnobacterium maltaromaticum (HP-B1)	+	-	+		Incompatible media	
Isolate G1-I3	-	-	-	P	-	P
Isolate G1-I7	-	-	-	P	-	-

Cell free culture supernatants of only two of these isolates displayed any quantifiable anti-*Vibrio* activity when tested using the method described in Section 3.2.1.2 (Figures 7-10). The isolates 'G-B' and 'HP-B1' were subsequently tentatively identified using the API 50 CHL (V5.1) sugar fermentation test as the lactic acid bacterium, *Carnobacterium maltaromaticum* (97.7% match). As was the case with *L. plantarum* and *P. acidilactici*, the cell free culture supernatants of *C. maltaromaticum* isolates were found to be strongly acidic (pH 4) and initially inhibited the growth of both *V. harveyi* and *V. alginolyticus* (Figures 8 & 9). This inhibition was not total,

however, with pathogen growth occurring after 12 h in the case of *V. harveyi* and 18 h in the case of *V. alginolyticus*. pH-attenuated cell free culture supernatant (pH 6.2) of *C. maltaromaticum* did not appear to interfere with the growth of *V. harveyi* but significantly reduced the growth rate of *V. alginolyticus* (Figure 8). Due to the lack of antagonistic activity displayed by the isolates 'G1-I3' & 'G1-I7' (Figures 9 & 10) only the suspected *C. maltaromaticum* was selected for further *in vivo* testing.

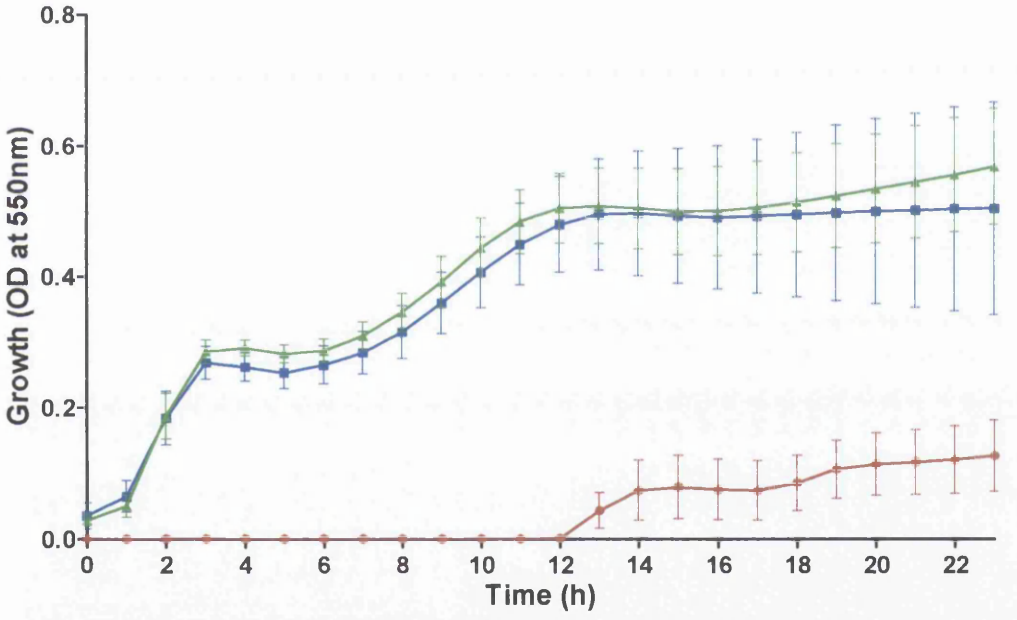


Figure 8. Growth profile of *Vibrio harveyi* in the presence of culture supernatant from suspected *Carnobacterium maltaromaticum*. *V. harveyi* and cell free supernatant of *C. maltaromaticum* at pH 4 (---), *V. harveyi* and cell free supernatant of *C. maltaromaticum* at pH 6.2 (---), *V. harveyi* and MRS broth only (---). Mean ± S.E.M, n=5.

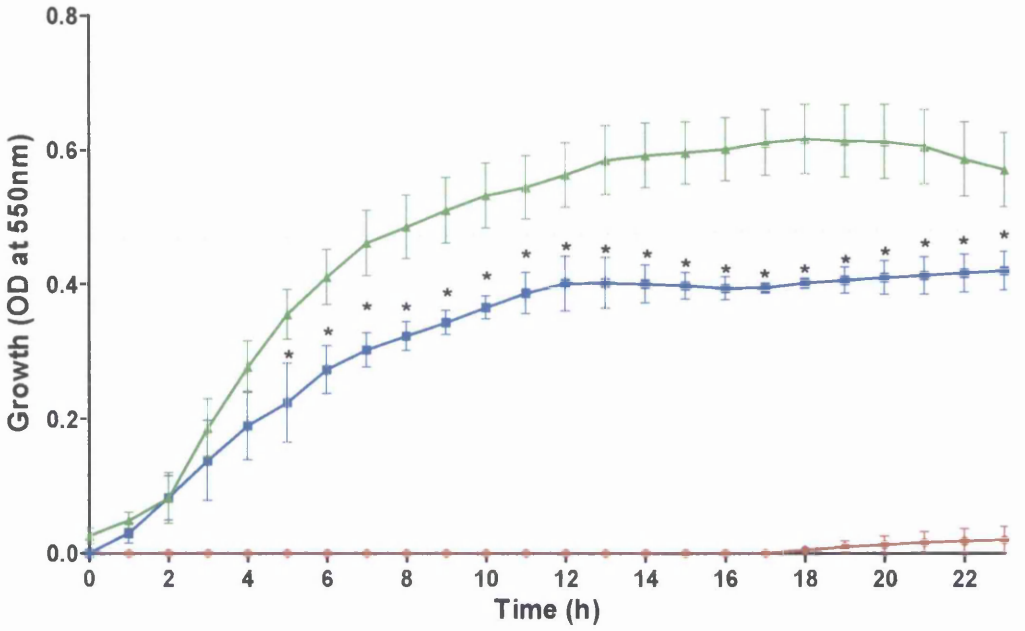


Figure 9. Growth profile of *Vibrio alginolyticus* in the presence of culture supernatant from suspected *Carnobacterium maltaromaticum*.*V. alginolyticus* and cell free supernatant of *C. maltaromaticum* at pH 4 (---), *V. alginolyticus* and cell free supernatant of *C. maltaromaticum* at pH 6.2 (---), *V. alginolyticus* and MRS broth only (---). Mean ± S.E.M, n=5, *P<0.05 compared to *V. alginolyticus* and cell free supernatant of *C. maltaromaticum* at pH 6.2.

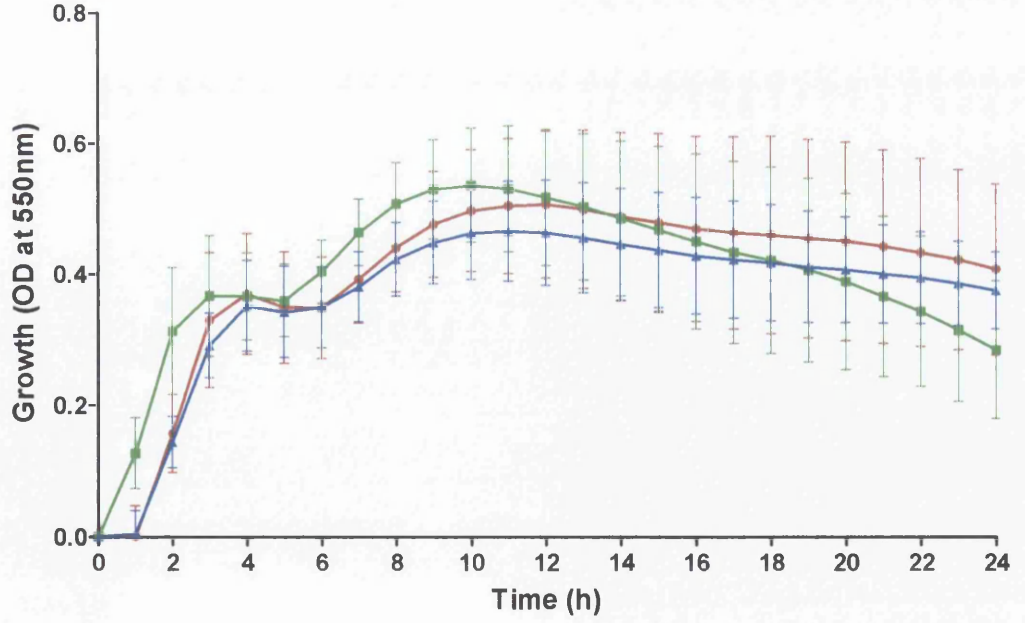


Figure 10. Growth profile of *Vibrio harveyi* in the presence of culture supernatant from shrimp isolates G1-I3 & G1-I7.*V. harveyi* and cell free supernatant of shrimp isolate G1-I7 (---), *V. harveyi* and cell free supernatant of shrimp isolate G1-I3 (---), *V. harveyi* and tryptic soy broth only (---). Mean ± S.E.M, n=3.

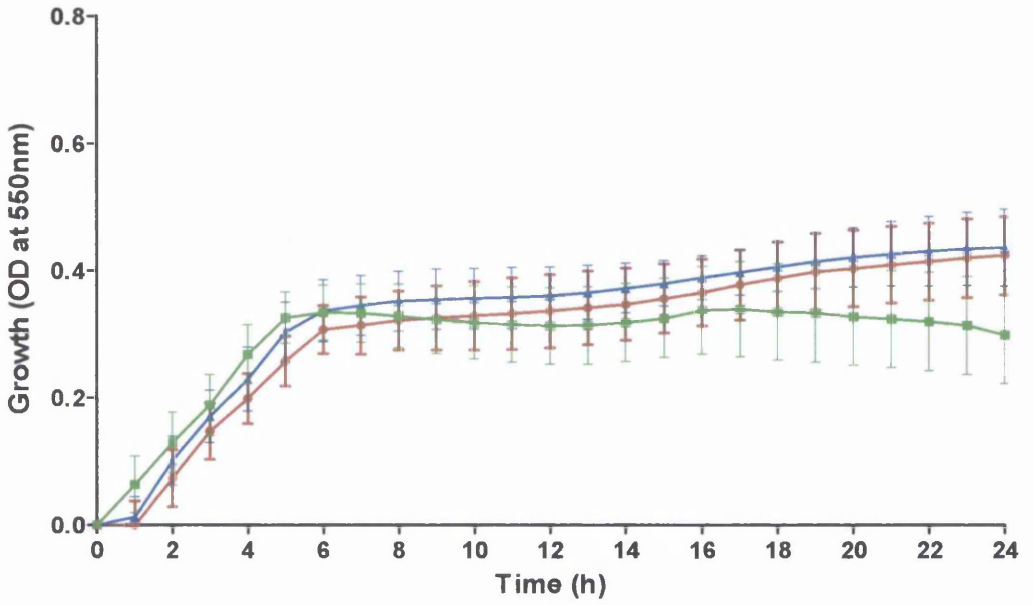


Figure 11. Growth profile of *Vibrio alginolyticus* in the presence of culture supernatant from shrimp isolates G1-I3 & G1-I7.*V. alginolyticus* and cell free supernatant of shrimp isolate G1-I7 (---), *V. alginolyticus* and cell free supernatant of shrimp isolate G1-I3 (---), *V. alginolyticus* and tryptic soy broth only (---). Mean ± S.E.M, n=3.

3.3.3 Screening of selected non-pathogenic *Vibrio* species and shell disease isolates for evidence of *in vitro* competitive inhibition of bacterial pathogens of crustaceans

Antagonistic activity toward selected crustacean pathogens was exhibited by two of the screened *Vibrio* species, *V. gazogenes* (NCIMB 2250) and *V. alginolyticus* (NCIMB 1339) (Tables 6 & 7; Figure 12A-D). No pathogen growth interference was noted for any of these test microbes' cell free culture supernatants (Table 6). Consequently, only *V. gazogenes* and *V. alginolyticus* were selected for further testing, *in vivo* (see Chapter 4).

Table 6. Antagonistic activity of selected *Vibrio* spp. against marine pathogens; no interference/inhibition (-), indication of interference/inhibition (+).

	NCIMB #	Antagonistic activity against pathogens					
		Cell free supernatant			Pathogen-isolate *in vitro* co-culturing		
		V. harveyi	*L. anguillarum*	*V. alginolyticus*	*V. harveyi*	*L. anguillarum*	*V. alginolyticus*
Listonella anguillarum	829	-		-	-		-
Vibrio alginolyticus	1339	-	-		+	+	
Vibrio gazogenes	2250	-	-	-	+	+	+
Vibrio harveyi	1280		-	-		-	-
Vibrio mediterranei	13228	-	-	-	-	-	-
Vibrio natriegens	2273	-	-	-	-	-	-
Vibrio orientalis	2195	-	-	-	-	-	-
Vibrio proteolyticus	1326	-	-	-	-	-	-
Vibrio scophthalmi	13623	-	-	-	-	-	-
Vibrio tubiashii	1336	-	-	-	-	-	-
SDI-1	-	-	-	-	-	-	-
SDI-4	-	-	-	-	-	-	-
SDI-8	-	-	-	-	-	-	-
SDI-9	-	-	-	-	-	-	-

Table 7. Antagonistic activity of *V. gazogenes* and *V. alginolyticus* against additional *Vibrio* pathogens; no interference/inhibition (-), indication of interference/inhibition (+).

	Antagonistic activity displayed during pathogen-isolate *in vitro* co-culturing				
	V. campbellii	*V. nigripulchritudo*	*V. parahaemolyticus*	*V. penaeicida*	*V. vulnificus*
Vibrio gazogenes	+	+	+	+	-
Vibrio alginolyticus	+	+	+	+	+

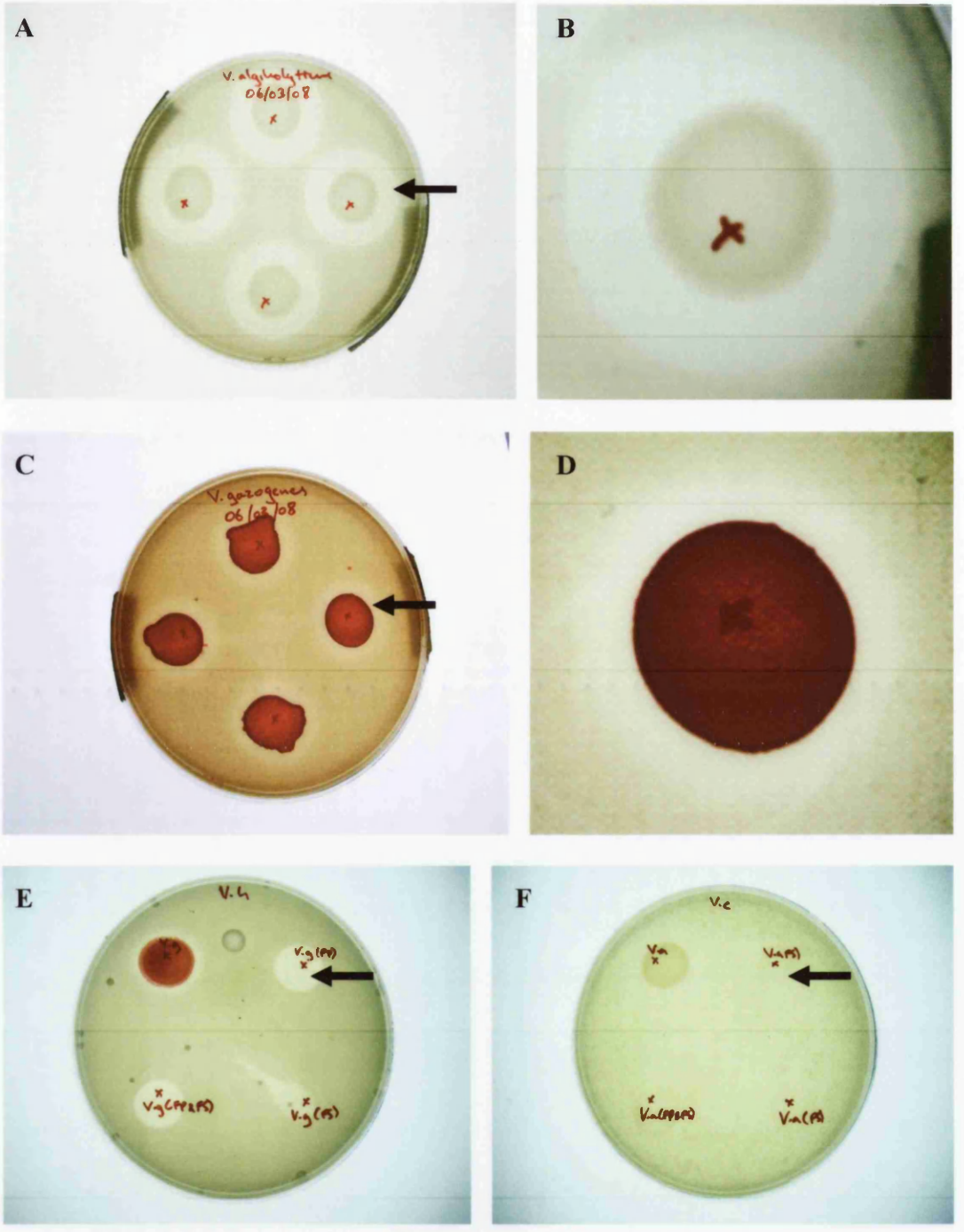


Figure 12. (**A**) *In vitro* co-culturing of *V. alginolyticus* against *V. harveyi*, with clear zone of inhibition of *V. harveyi* growth indicated by arrow; also (**B**) close up view of area of growth inhibition. (**C**) *In vitro* co-culturing of *V. gazogenes* against *V. harveyi*, with zone of inhibition of *V. harveyi* growth indicated by arrow; also (**D**) close up view of area of growth inhibition. (**E**) Plate displaying antagonistic activity of French pressed (killed) *V. gazogenes* culture material against *V. harveyi* (indicated by arrow). (**F**) Plate displaying absence of antagonistic activity of French pressed (killed) *V. alginolyticus* culture against *V. campbellii* (indicated by arrow).

French pressed culture material of *V. gazogenes* (i.e. lysed bacteria) retained its antagonistic activity (in the form of growth inhibition/interference) towards all screened pathogens with the exception of *L. anguillarum* and *V. penaeicida* (Table 8 & Figure 12E). Activity displayed was lower than that of live culture *V. gazogenes*; however, the material retained its activity despite filter sterilisation. The French pressed culture material of *V. alginolyticus* displayed no such anti-*Vibrio* activity (Table 8 and Figure 12F).

Table 8. Antagonistic activity of French pressed *V. gazogenes* and *V. alginolyticus* cultures against *Vibrio* pathogens; no interference/inhibition (-), indication of interference/inhibition (+).

Crustacean pathogen	NCIMB #	*In vitro* antagonistic activity displayed during co-culturing	
		Vibrio alginolyticus	*Vibrio gazogenes*
L. anguillarum	829	-	-
V. alginolyticus	1339		+
V. campbellii	1894	-	+
V. harveyi	1280	-	+
V. nigripulchritudo	1904	-	+
V. parahaemolyticus	1164	-	+
V. penaeicida	13386	-	-
V. vulnificus	2046	-	+

3.4 Discussion

The three sources of micro-organisms yielded five potentially probiotic bacterial strains for further testing; *Lactobacillus plantarum*, *Pediococcus acidilactici*, suspected *Carnobacterium maltaromaticum*, *Vibrio alginolyticus* and *Vibrio gazogenes*. All five exhibited antagonistic activity towards selected potential crustacean bacterial pathogens, either in the form of inhibitory compounds released into the culture media (the three lactic acid bacteria) or via direct competitive inhibition (*V. alginolyticus* and *V. gazogenes*). There are numerous potential mechanisms by which bacteria can inhibit the growth of competing microorganisms. For example, in the case of some *Vibrio* spp. (e.g. *V. harveyi*) it takes the form of the production of bacteriocin-like inhibitory substances (BLIS) (Prasad et al, 2005). Most species of lactobacilli are capable of producing hydrogen peroxide, which can be toxic to bacteria incapable of producing H_2O_2 scavenging enzymes (Eschenbach et al, 1989). Another potential factor involved in (growth) competition within the microbiota is the requirement for iron, an element vital for the growth of the majority of bacteria (Dhungana et al 2007). Lactic acid bacteria (in particular *Lactobacillus plantarum* ATCC 14917) show no decline in growth or proliferation rates when subjected to iron deprivation (Pandey et al, 1994). A characteristic of most bacterial pathogens is the possession of highly efficient haem uptake mechanisms in order to provide them with iron for growth within host body fluids and tissues, an ability directly linked to their virulence (Stojiljkovic et al, 1999). Therefore, any non-pathogenic species unaffected by iron deprivation could theoretically have a major competitive advantage over such pathogens and could be regarded as a potential probiont. On an additional note the production of the red pigment, prodigiosin, by *V.*

gazogenes was a further useful characteristic in allowing the easy identification of the potential probiotic in samples/assays (Allen et al, 1983).

The main active component in the inhibitory activity displayed by the lactic acid bacteria was pH, as a result of the release of lactic acid into the microbe's immediate environment. In addition to this, however, there appeared to be a secondary component(s) to the displayed inhibition. The pH-adjusted cell-free culture supernatant of all three strains significantly inhibited the growth of *V. alginolyticus* compared to the bacteria-only controls. The inhibition exhibited was considerably reduced compared to that of the original supernatants, but the presence of a secondary inhibitory component was indicated. Indeed, strains of all three species have been found to produce antimicrobial peptides or bacteriocins in addition to lactic acid (Suma et al 1998; Verellen et al, 1998; Blom et al, 2001; Calderón-Santoyo et al, 2001; Jamuna & Jeevaratnam, 2004; Gursky et al, 2006; Martin-Visscher et al, 2008). The cell-free culture supernatant of suspected *C. maltaromaticum* displayed less inhibitory ability toward potential pathogens than that of *L. plantarum* or *P. acidilactici*. Despite this, the fact that it is a novel strain, isolated from the host species, makes it worthy of further testing. Whether suspected *C. maltaromaticum*, *L. plantarum* and *P. acidilactici* cultures can maintain their inhibitory potential *in vivo* and benefit the host animal is the key question. There is little likelihood of these micro-organisms being able to reduce the pH of the G.I. tract of a crustacean host to a level where other potentially pathogenic bacteria are inhibited. If this were possible, however, such a reduced pH may in fact be detrimental to the host, e.g. causing a reduction in digestive efficiency.

With regard to the anti-*Vibrio* activity of *V. alginolyticus*, the lost of activity resulting from the killing of the bacterium indicates that the inhibitory ability of this micro-

organism is solely reliant on the presence of live cells. This may indicate that the components of inhibition are either extremely short-lived (i.e. they breakdown rapidly) and therefore need to be constantly produced, or are associated with cell structures such as the cell wall/membrane. The inhibitory ability of *V. gazogenes*, however, does not rely on the presence of live cells. The French pressed and filter sterilised *V. gazogenes* whole broth culture still retained anti-*Vibrio* activity, although less than that observed for the live culture. Given that numerous strains of *V. alginolyticus* exhibit pathogenicity toward crustaceans (Lee et al, 1996; Liu et al, 2004; Wang & Chen, 2005; Jayasree et al, 2006) coupled with the fact that any supplemented feed would need to contain live cells, makes it unlikely that this strain of *V. alginolyticus* would ever be licensed for use in aquaculture feedstuffs. For completeness, however, the pathogenicity of this strain was assessed along with the suspected *C. maltaromaticum* and *V. gazogenes* in Chapter 4.

The screening strategy adopted in this study focussed purely on the ability of the test micro-organisms to directly interfere with/inhibit the growth of potential crustacean pathogens. This, as stated in greater detail in Chapter 1, is only one of several possible modes of action of a potential probiotic bacterium (Balcázar et al, 2006). The production of inhibitory substances (against pathogenic bacteria) is, however, the most widely studied and well documented mode of probiotic action (Kesarcodi-Watson et al, 2008). The stages of isolation and screening followed by *in vivo* pathogenicity testing of isolates exhibiting inhibitory activity are part of a well established methodology for the identification of potential novel probiotics (Decamp & Moriarty, 2006; Kesarcodi-Watson et al, 2008). The battery of assays employed in this chapter were all optimised from widely used methods for the screening of bacteria for the production of inhibitory compounds and competitive interactions

(Pilet et al, 1995; Gram et al, 1999; Sambrook & Russell, 2001; Hjelm et al, 2004). Ideally, additional approaches would have been undertaken to investigate the isolates for other probiotic modes of action, however, this was not possible within the scope of the project. In addition the examination of other bacterial groups, in particular members of the Bacillaceae, is another aspect worthy of further exploration.

Chapter 4

Safety assessment of potential crustacean probiotics; the determination of the pathogenicity of suspected *Carnobacterium maltaromaticum*, *Vibrio gazogenes* and *Vibrio alginolyticus* towards the Pacific white shrimp, *Litopenaeus vannamei*

Abstract

Any micro-organism intended for use in consumer products and feedstuffs destined for human or animal consumption, must be assessed with regards to the potential danger they may pose to the host. The benefits conveyed via the administration of such micro-organisms must outweigh any negative effects. Bacterial strains displaying unacceptable levels of pathogenicity towards a host are hence unsuitable for use as probiotics. The pathogenicity of three selected micro-organisms towards the Pacific white shrimp, *Litopenaeus vannamei*, were assessed via the intramuscular injection of live bacteria. These micro-organisms were; *Vibrio alginolyticus* (NCIMB 1339), *Vibrio gazogenes* (NCIMB 2250) and a suspected strain of *Carnobacterium maltaromaticum* isolated from cultured *L. vannamei*. The mortalities incurred were recorded and tissue samples retained for histopathology. *V. alginolyticus* displayed unacceptably high levels of pathogenicity towards *L. vannamei* and was therefore discarded as a potential probiotic. Both *V. gazogenes* and suspected *C. maltaromaticum* displayed some pathogenicity towards *L. vannamei*, but considerably less than that of *V. alginolyticus*. Suspected *C. maltaromaticum* was, however, disregarded as a candidate probiotic due to its lower anti-bacterial activity compared to other candidate lactic acid bacteria and its colonisation of the culture system biofilm. The *V. gazogenes* strain was selected for further dietary testing (alongside the two commercial species of lactic acid bacteria) due to its mode of anti-bacterial activity and low level of pathogenicity towards *L. vannamei*.

4.1 Introduction

Prior to oral administration of new potentially probiotic bacterial strains to an aquaculture species, particularly those intended for human consumption, it is prudent to assess the pathogenicity of that micro-organism (in the event of infection) towards the host species. Any bacterial isolate displaying significant pathogenicity towards its target host species would be unmarketable as a commercial supplement, regardless of its probiotic potential.

It was regarded as unnecessary to subject *L. plantarum* and *P. acidilactici* used in Chapters 2 & 3 to such testing since they are already approved as probiotic supplements in animal agricultural. Both micro-organisms are currently listed as authorised probiotics in animal feeding stuffs within the European Union (European Commission, 2004 - Community Register of Feed Additives; pursuant to regulation (EC) No 1831/2003 Rev. 44; Balcázar et al, 2006). *P. acidilactici* is also 'generally recognised as safe' (G.R.A.S.) by the United States Food & Drug Administration (Salminen et al, 1998) and both have been regarded as having 'Qualified Presumption of Safety' (Q.P.S) status by the European Food Safety Authority (EFSA). Therefore, the likelihood of either of these species being sufficiently pathogenic toward *L. vannamei* was deemed low enough for them to proceed directly to the feed trial stage. Indeed once a micro-organism is deemed safe, e.g., awarded QPS status by the EFSA or GRAS status by the FDA, no further assessment of safety for the target species, the consumer and the wider environment is required by that body, only of the commercial product's efficacy (Sanders et al, 2007). Consequently, this chapter covers the *in vivo* challenge of *L. vannamei*, via intramuscular injection, by suspected *C. maltaromaticum*, *V. gazogenes* and *V. alginolyticus*.

While no specific guidelines exist with regard to the trial criteria/test dosages involved in the pathogenicity assessment of potential probiotics, there is a general consensus that such an assessment is a requirement (Verschuere et al, 2000; Decamp & Moriarty, 2006; Sanders et al, 2007; Kesarcodi-Watson et al, 2008). The licensing of microbial animal feed additives for use within the E.U requires the producer to provide evidence of safety, i.e. to show qualified presumption of safety (Q.P.S) (von Wright, 2005; Balcázar et al, 2006). Despite this, the European Commission legislation (Council Directive 70/524/EEC) and the agency (European Food Safety Authority) responsible for the regulation of these feed additives merely lists a series of 'opinions' suggesting that potential probiotic strains should be non-pathogenic and non-toxic, but defines no specific safety parameters (European commission, 2003; Directorate C – Scientific opinions, "On a generic approach to the safety assessment of micro-organisms used in feed/food and feed/food production"). Human probiotic foods, however, are not governed under any specific European Commission regulatory framework (von Wright, 2005). Consequently, the dosages used were derived from literature on previous *Lactobacillus* safety assessments and those indicating the LD_{50} doses of pathogenic Vibrionaceae (Lara-Villoslada et al, 2007; Liu et al, 2004; Phuoc et al, 2009).

The aims of this chapter were:

- To assess the degree of pathogenicity displayed by suspected *C. maltaromaticum*, *V. gazogenes* and *V. alginolyticus* toward the crustacean target host, *L vannamei.*

- To determine which of the above should be selected, alongside *L. plantarum* and *P. acidilactici*, for later *in vivo* feed trials in *L vannamei* (Chapter 5).

4.2 Materials & Methods

4.2.1 Assessment of pathogenicity of potential probiotics suspect *C. maltaromaticum, V. gazogenes* and *V. alginolyticus* towards Pacific white shrimp *L. vannamei*

Post-larval Pacific white shrimp, *L. vannamei*, obtained from the CSAR facility at Swansea University were subjected to *in vivo* challenge by suspected *C. maltaromaticum, V. gazogenes* (NCIMB 2250) and *V. alginolyticus* (NCIMB 1339). Challenges were conducted to determine the degree of pathogenicity the organisms displayed towards a crustacean host and thus their viability as potential probiotics. The suspected *C. maltaromaticum* isolate and *V. gazogenes* were regarded as being sufficiently safe for testing within a small closed system at the CSAR facility (Swansea University). Suspected *C. maltaromaticum* was originally isolated from a healthy animal raised in one of the CSAR systems; consequently, it was regarded as posing little danger to the resident *L. vannamei* population. *V. gazogenes* has no history of pathogenicity towards any marine species and has been isolated from the gastrointestinal microflora of healthy, wild and cultured Banana prawn, *Penaeus merguiensis* (Oxley et al, 2002). However, given that several strains of *V. alginolyticus* are pathogenic to shrimp (Selvin & Lipton, 2003; Wang & Chen, 2005; Li et al, 2008), coupled with the possibility of contamination of the main *L. vannamei* culture system, it was decided that *V. alginolyticus* (NCIMB 1339) required testing in a system that could be sterilised, such as those employed at the CEFAS research facility, Weymouth, UK.

4.2.1.1 *Vibrio alginolyticus* (NCIMB 1339) challenge

Three treatment groups each containing 12 animals (*L. vannamei*; 4 ± 1 g) were housed in 3 x 40 l tanks at the CEFAS research facility, Weymouth, UK. Animals of the first group, designated the control, were administered an injection of 100 µl sterile 3% NaCl solution, intramuscularly, between the second and third pleomeres. The remaining groups were injected with an equal volume of suspensions of *V. alginolyticus*. These suspensions comprised a low dose of *ca.* 3 x 10^6 total cells ml^{-1} and a high dose of *ca.* 3 x 10^8 total cells ml^{-1} modified from the methods of Liu et al (2004). *V. alginolyticus* was cultured in TSB (plus 2% NaCl) at 25°C for 24 h prior to centrifuging (1000 g 5 min at 25°C). The resulting pellet was re-suspended in 1 ml sterile 3% NaCl solution and its concentration adjusted to provide the required suspensions.

No feed was administered during the trial, the animals were checked at 1, 18, 24, 30 & 44 h post-injection with all mortalities removed and recorded. Three moribund animals from the high dose group were sampled for histological examination at 1 h post administration. At 24 h post-administration; two live animals from the low dose group were sacrificed for tissue samples (none of the high dose group survived at this point). The trial was ended at 44 h with no further mortality observed in the control or low dose groups post 18 h. At this point, 2 animals from both groups were sacrificed for tissue samples. All live animals sampled were killed via injection of *ca.* 5 ml of Bouin's seawater fixative. Thoracic and abdominal sections (containing the site of injection) of each animal were retained in excess volume of fixative, at room temperature for later histopathology (Section 4.2.1.4). An estimate of the 50%

endpoint of the challenge (estimated 50% lethal dose; est. LD_{50}) was determined using the method outlined in Reed & Muench (1938).

4.2.1.2 *Vibrio gazogenes* (NCIMB 2250) challenge

Three treatment groups each containing 20 animals (*L. vannamei* 4 ± 1 g) were housed over 6 x 30 l tanks of a closed, re-circulating system at the CSAR facility, Swansea University. Animals of the first group, designated the control, were administered an inoculation of 100 µl sterile 3% NaCl solution, intramuscularly, between the second and third pleomeres. The remaining groups were injected with an equal volume of suspensions of *V. gazogenes*. These suspensions comprised a low dose of *ca.* 3 x 10^6 total cells ml^{-1} and a high dose of *ca.* 3 x 10^8 total cells ml^{-1} modified from the methods of Liu et al (2004). *V. gazogenes* was cultured in TSB (plus 2% NaCl) at 25°C for 24 h prior to centrifuging (1000 g 5 min at 25°C). The resulting pellet was re-suspended in 1 ml sterile 3% NaCl solution and its concentration adjusted to provide the required suspensions.

No feed was administered during the trial, however, the animals were checked at 24 h intervals, with all mortalities removed and recorded. At 12 & 24 h post administration; two animals from each group were sacrificed for tissue samples. In addition, after 24 h those animals that had received *V. gazogenes* (once euthanised on ice) were transversely bisected at the join between the first and second abdominal segments (pleomeres). The cut surface was repeatedly touched onto two TSA (plus 2% NaCl) plates, these were incubated at 25°C (24 h) before being checked for *V. gazogenes* growth (*V. gazogenes* colonies produce a distinctive pink/red pigmentation on TSA). Thoracic sections (containing gill and hepatopancreas) and abdominal

sections (containing the site of injection) were retained in excess volume of Bouin's seawater fixative, at room temperature for histopathology. The samples retained for histopathology were processed and examined as stated in Section 4.2.1.4. The trial was terminated after 6 days due to a lack of mortality across the groups in the preceding 72 h. Twenty four hours after the cessation of the trial, samples of tank biofilm were taken and plated (TSA plus 2% NaCl) to ascertain whether *V. gazogenes* had become a component of the system microbiota. An estimate of the 50% endpoint of the challenge (estimated 50% lethal dose; est. LD_{50}) was determined using the method outlined in Reed & Muench (1938).

4.2.1.3 Suspect strain *Carnobacterium maltaromaticum* challenge

Post larval *L. vannamei* weighing 1 ± 0.2 g (two groups of 10 animals) were housed in two, 30 l tanks within a closed re-circulating system at the CSAR Tropical quarantine facility, Swansea University. Animals of the first group, designated the control, were administered an intramuscular injection of 100 µl sterile 3% NaCl solution, intramuscularly between the second and third pleomeres. The remaining group was injected with equal volume of a suspension of suspect strain *C. maltaromaticum.* Suspect strain *C. maltaromaticum* was cultured in MRS broth (plus 2% NaCl) at 25°C for 24 h before centrifuging (1000 g 5 min at 25°C). The pellet was re-suspended in 1 ml sterile 3% NaCl solution and its concentration adjusted to *ca.* 1×10^8 cells ml^{-1} (Lara-Villoslada et al, 2007).

No feed was administered during the trial, however, the animals were monitored at 12 h intervals, with any mortalities removed and recorded. At 24, 72 and 96 h post-

injection; two shrimp from each group were sacrificed for haemolymph and histology. The size of the animals made obtaining a haemolymph sample via needle impossible. Consequently, the animals (once euthanised on ice) were transversely bisected at the join between the cephalothorax and first abdominal segment (pleomere). The cut surface of the abdominal section was touched onto two MRS agar (plus 2% NaCl) plates, the *ca.* 50 µl of haemolymph deposited was aseptically spread and the plates incubated at 25°C (72 h) before being checked for growth. The highly selective nature of MRS media toward lactic acid bacteria, combined with the low abundance of such bacteria in the microflora of *L. vannamei* (Section 3.2.2 & Appendix 3) the majority of colonies recovered can be regarded as belonging to suspect strain *C. maltaromaticum.* Given that the volume of haemolymph plated was unknown, CFU ml^{-1} could not be determined; hence data were recorded as CFU per plate. Abdominal sections of both control and infected animals (around the site of injection) were retained in an excess volume of Bouin's seawater fixative (Appendix 1) at room temperature for histopathology. These tissue samples were processed and examined as stated in Section 4.2.1.4. The trial was ended after 96 h due to high cumulative mortality within the group administered the suspect strain *C. maltaromaticum.* 24 h after the cessation of the trial samples of tank biofilm were taken and plated (MRS agar plus 2% NaCl) to ascertain whether suspected *C. maltaromaticum* had become a component of the system microbiota.

4.2.1.4 Histopathology

The fixed tissue samples were dehydrated using progressive, graded alcohol washes (70, 80, 90 and 100% analytical grade ethanol) 1 h per grade. Tissues were then

cleared via two immersions (4 h total) in Histoclear (Fisher Scientific Ltd, Leicestershire, UK). Samples were then submerged in molten paraffin wax (3 changes) for 6 h, before embedding in wax. After a minimum of 24 h (to ensure adequate hardening of the wax) 10 µm sections were cut and stained using Cole's haematoxylin and eosin (Gretchen & Humason, 1979) (Appendix 1). Photomicrographs were taken using an Olympus BX50 binocular microscope and digital camera (Olympus Optical, London, UK).

4.3 Results

4.3.1 *Vibrio alginolyticus* (NCIMB 1339) challenge

Vibrio alginolyticus (NCIMB 1339) displayed a high degree of pathogenicity towards juvenile *L. vannamei* (Figure 1). The high dose group (*ca.* 3 x 10^7 bacteria shrimp^{-1}) experienced 50% mortality within 1 h of administration and 100% mortality within 18 h. The low dose group (*ca.* 3 x 10^5 cells shrimp^{-1}) displayed a cumulative mortality of 29% at the cessation of the trial. The data collected allowed the determination of an estimate of the 50% endpoint of the trial and an estimated LD_{50} of *ca.* 9.1 x 10^6 bacteria shrimp^{-1}.

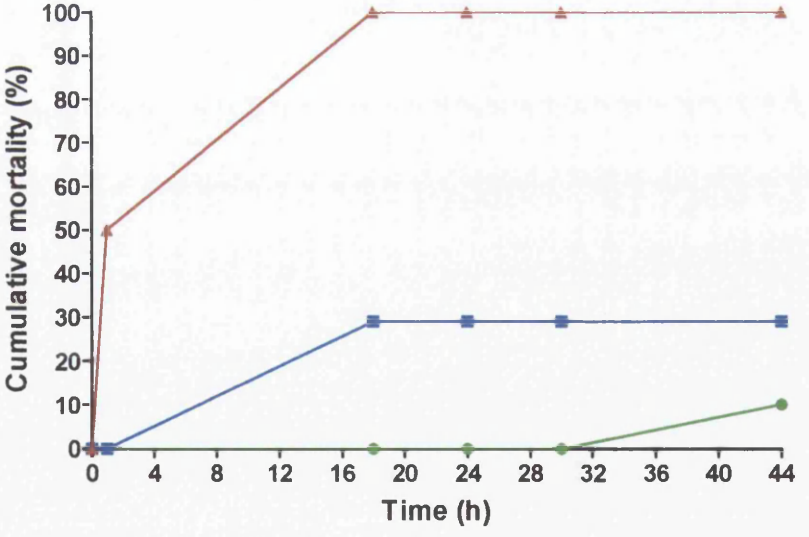


Figure 1. Cumulative mortality of Pacific white shrimp, *Litopenaeus vannamei*, over 44 hr post administration of *ca.* 3 x 10^7 cells shrimp^{-1} of *Vibrio alginolyticus* (---), *ca.* 3 x 10^5 cells shrimp^{-1} of *Vibrio alginolyticus* (---) or 3% NaCl solution control (---).

Histological examination of gill samples from moribund animals of the high dose group (1 h post administration) displayed early nodule formation in the primary blood

vessels of the gill lamellae (Figures 2A-D). Samples of muscle from the area surrounding the injection site (high dose group) displayed no such histopathology. No evidence of nodule formation in the gills was noted in samples from the low dose group, however, samples from 44 h showed evidence of haemocyte infiltration in muscle tissue in the vicinity of the injection site (Figure 3A & B). *V. alginolyticus* was not recovered from any of the samples taken of the culture system's biofilm.

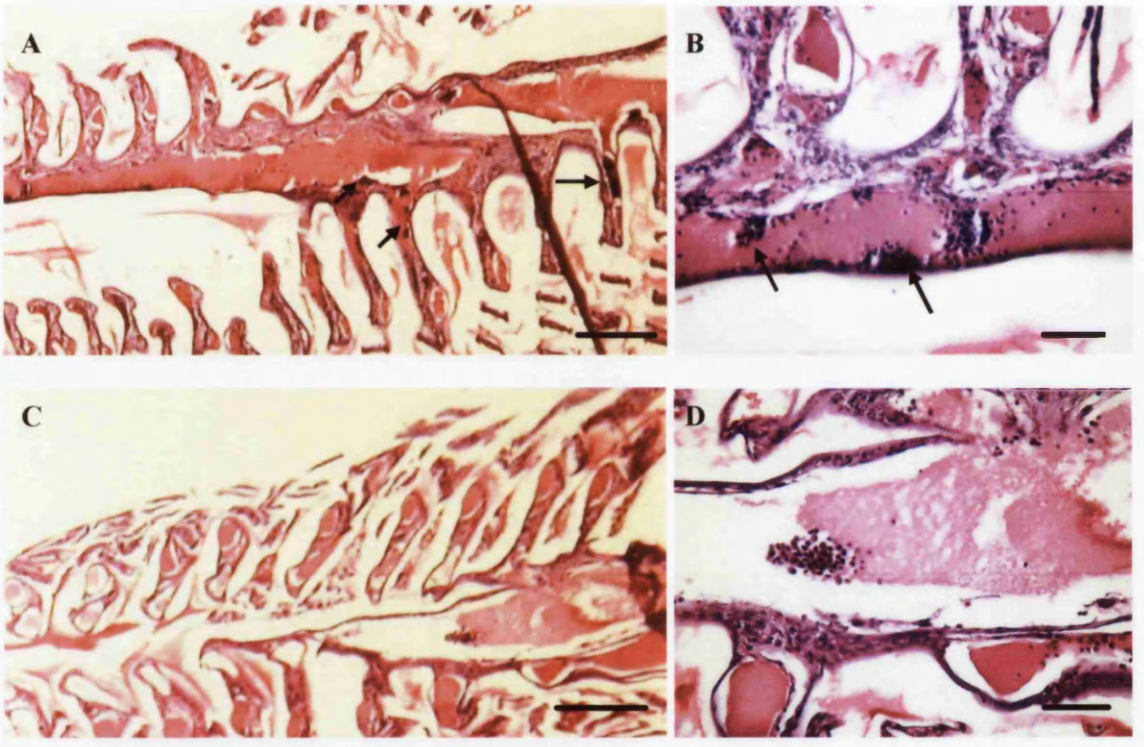


Figure 2. (**A**) Low power micrograph of a transverse section of gill lamellae from *Litopenaeus vannamei* 1 h after receiving high dose of *V. alginolyticus*, arrows indicating haemocyte nodule formation (Scale bar = 250 µm). (**B**) Mid power view of a transverse section of gill lamellae of *Litopenaeus vannamei* 1 h after receiving high dose of *V. alginolyticus*, arrows indicating early haemocyte nodule formation (Scale bar = 100 µm). (**C**) Low power micrograph of a transverse section of gill lamellae of *Litopenaeus vannamei* 1 h after receiving saline, displaying absence of haemocyte nodules (Scale bar = 250 µm). (**D**) Mid power view of a transverse section of gill lamellae of *Litopenaeus vannamei* 1 h after receiving saline, displaying absence of haemocyte nodules (Scale bar = 100 µm).

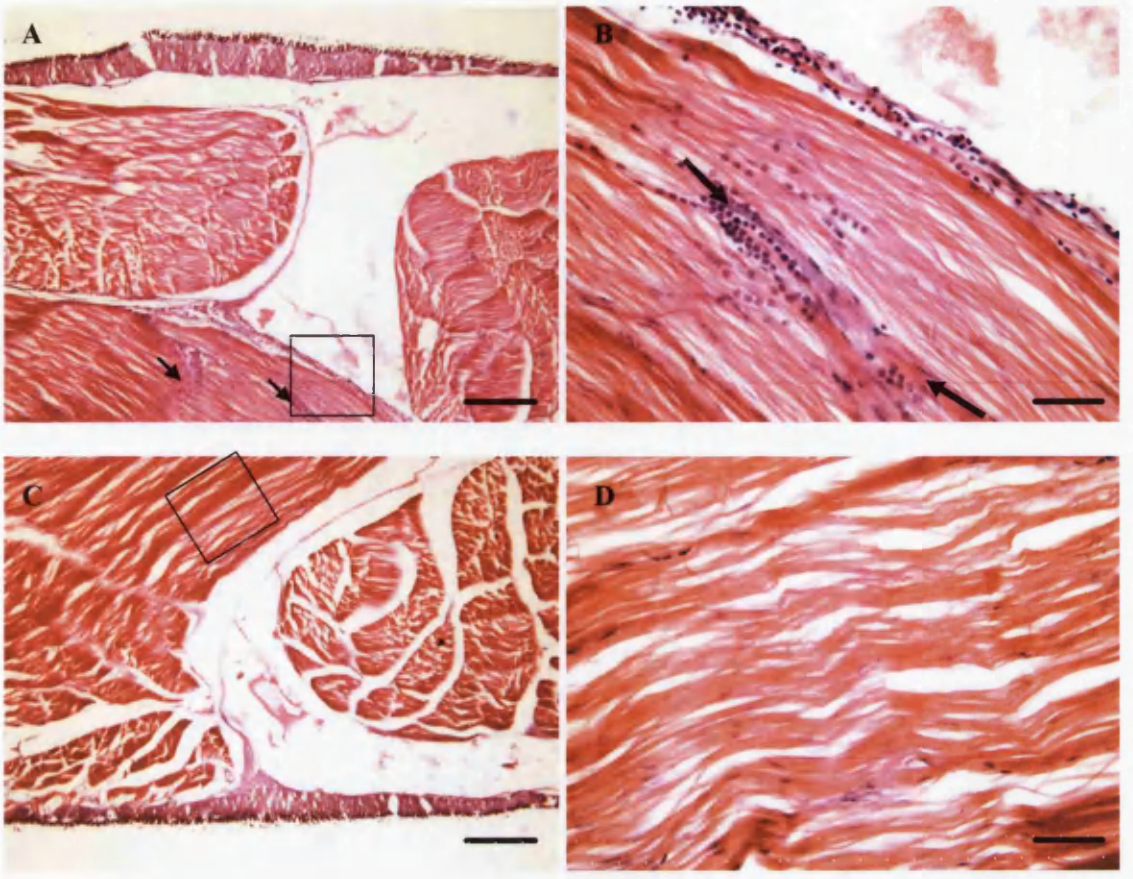


Figure 3. (**A**) Low power micrograph of a transverse section of the abdominal region of *Litopenaeus vannamei* displaying muscle and external cuticle 44 h after receiving low dose of *V. alginolyticus*. The area shown is in the immediate vicinity of the injection site. Arrows indicate regions of haemocyte infiltration of the muscle tissue (Scale bar = 200 µm). (**B**) Mid-power micrograph displaying the enclosed area in (**A**), arrows indicate regions of haemocyte infiltration of the muscle tissue (Scale bar = 50 µm). (**C**) Low power micrograph of a transverse section of the abdominal region of *Litopenaeus vannamei* displaying muscle and external cuticle 44 h after receiving low dose of *V. alginolyticus*. Area shown is on the opposite side of the body to the site of injection; i.e. displays no indication of haemocyte infiltration of the muscle (Scale bar = 200 µm). (**D**) Mid power micrograph displaying the enclosed area in (**C**), exhibiting no evidence of haemocyte infiltration (Scale bar = 50 µm).

4.3.2 *Vibrio gazogenes* (NCIMB 2250) challenge

Vibrio gazogenes (NCIMB 2250) exhibited pathogenicity towards *L. vannamei* only when administered at the high dose of, *ca.* 3 x 10^7 total bacteria shrimp^{-1} (Figure 4). No mortality was observed in the control or low dose groups during the 144 h observation period. In the high dose group, a cumulative mortality of 94% was recorded, lower than that for *V. alginolyticus* under the same trial conditions. The mortality data allowed for the determination of an estimate of the 50% endpoint of the trial and an estimated LD_{50} of *ca.* 1.6 x 10^7 bacteria shrimp^{-1}.

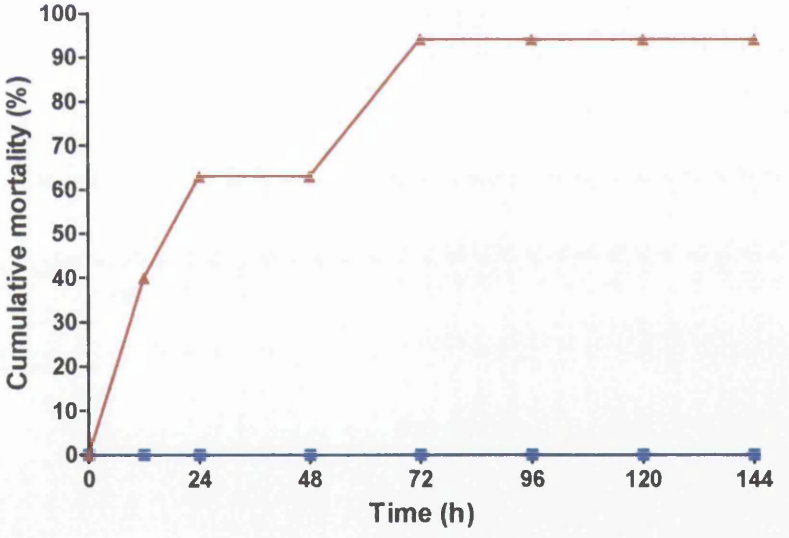


Figure 4. Cumulative mortality of Pacific white shrimp, *Litopenaeus vannamei*, over 148 hr post administration of *ca.* 3 x 10^7 bacteria shrimp^{-1} of *Vibrio gazogenes* (---), *ca.* 3 x 10^5 bacteria shrimp^{-1} of *Vibrio gazogenes* (---) or 3% NaCl solution control (---).

The presence of live *V. gazogenes* in the tissues surrounding the site of injection was confirmed in all high dose animals sacrificed at 24 h, via touch plating (Figure 5).

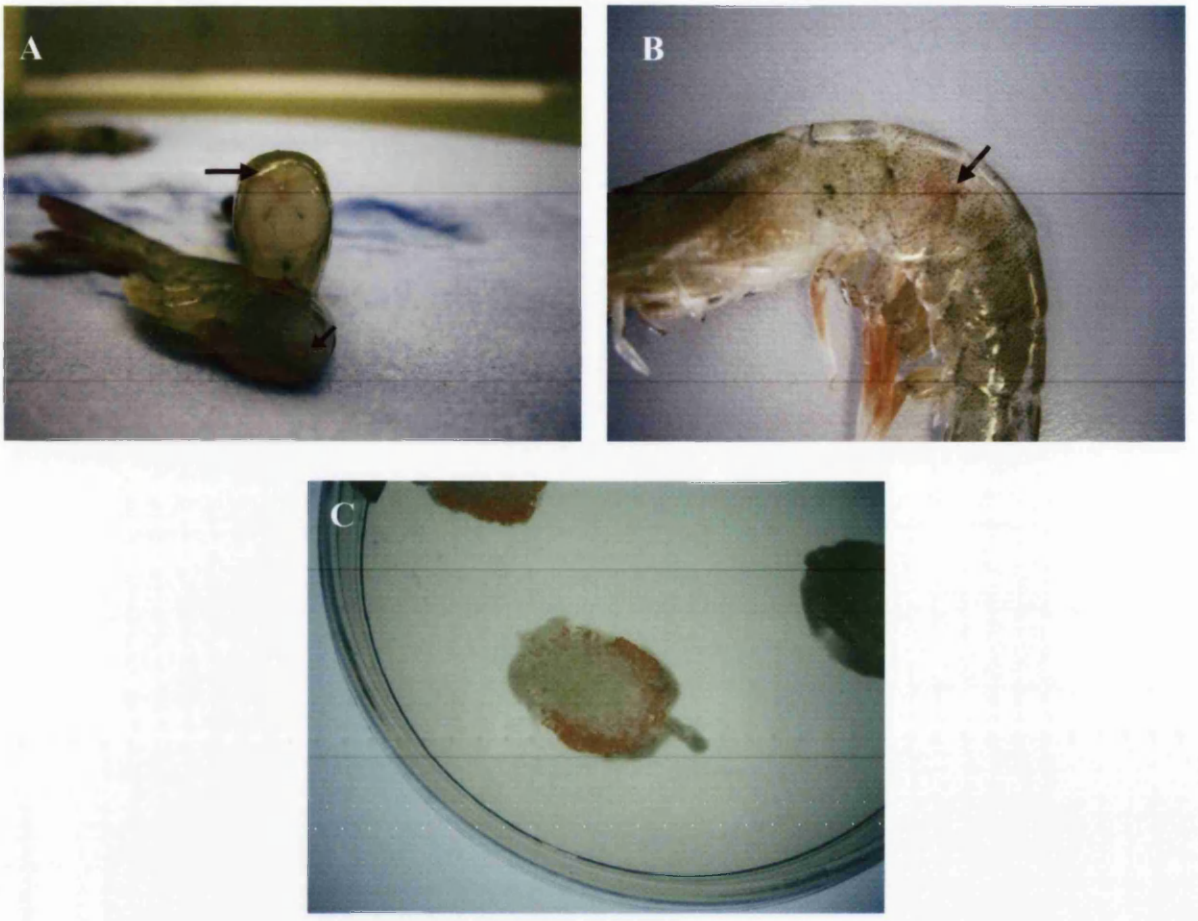


Figure 5. (**A**) Transversely bisected juvenile *Litopenaeus vannamei* sacrificed 24 h post-administration of *ca.* 3 x 10^7 *Vibrio gazogenes* (NCIMB 2250) shrimp^{-1}. Arrows indicate localised presence of *V. gazogenes* (exhibiting pink pigmentation). (**B**) External view of a juvenile *Litopenaeus vannamei* sacrificed 24 h post-administration of *ca.* 3 x 10^7 cells shrimp^{-1} of *Vibrio gazogenes* (NCIMB 2250); arrow indicates localised presence of *V. gazogenes* (exhibiting pink pigmentation). (**C**) Plate inoculated with haemolymph from animal shown in (**A**) via touch plating, clearly indicating presence of viable *V. gazogenes* (areas of red pigmentation).

Histological sections from animals in the high dose group displayed evidence of localised muscle necrosis in the region surrounding the injection site, but no evidence of pathology in the gills. No evidence of haemocyte nodule formation was observed in the blood vessels of the gill lamellae; which would have been indicative of the early

stages of a systemic bacterial infection (Figure 6). Necrotised muscle tissue, localised around the site of injection was observed in abdominal sections from animals in the high dose group (Figures 7A & B). In addition, evidence was found indicating the possible presence of a secondary yeast-like infectious agent (Figure 8A). No such pathology was observed in the animals administered the lower dose of *V. gazogenes*. However, the presence of what appeared to be bacteria was noted within the muscle of one animal administered the low dose of *V. gazogenes* (Figure 8B).

V. gazogenes was not isolated from any samples taken of the culture system's biofilm.

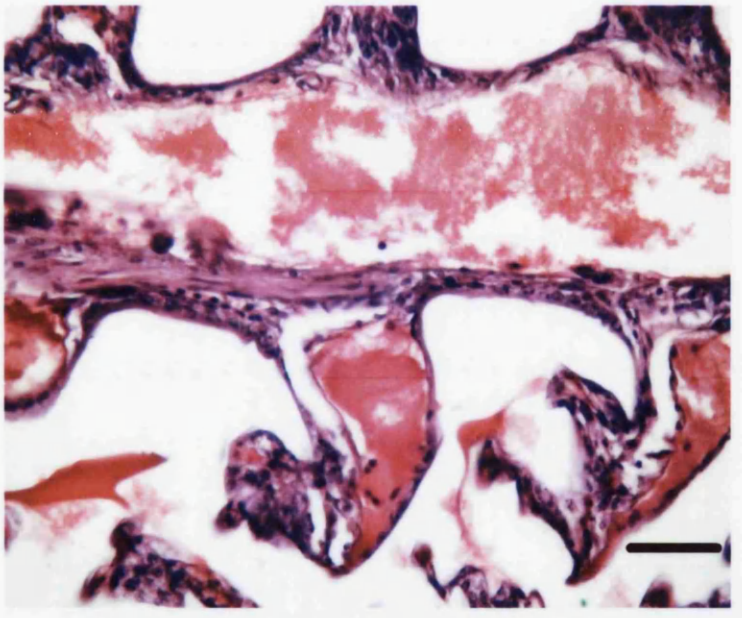


Figure 6. Low power micrograph of a transverse section of gill lamellae taken from *L. vannamei* sacrificed 24 h post-administration of *ca.* 3 x 10^7 *Vibrio gazogenes* shrimp^{-1}, showing no indication of haemocyte nodule formation observed (Scale bar = 100 μm).

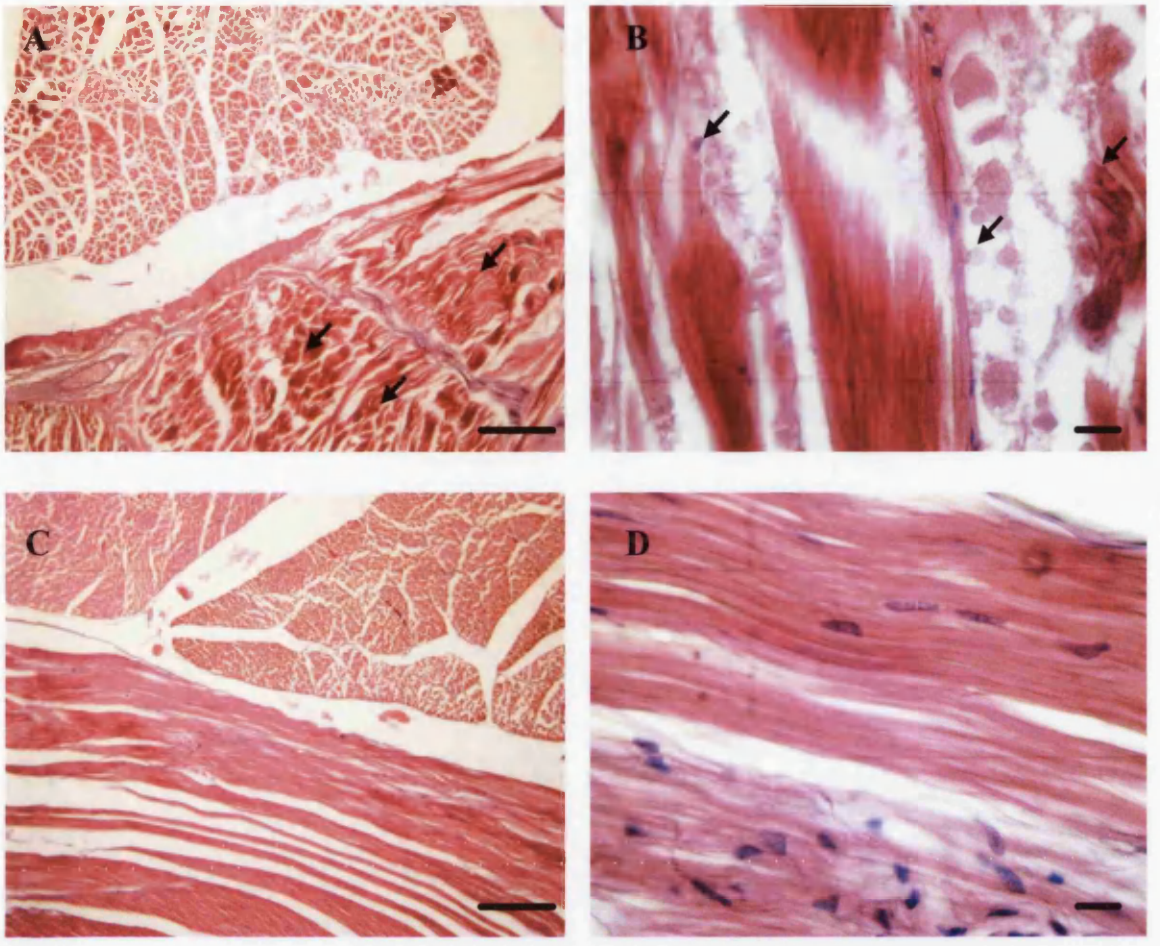


Figure 7. (**A**) Low power micrograph of a transverse section of the abdominal region of *Litopenaeus vannamei* showing muscle 12 h after receiving the high dose of *V. gazogenes*. Area shown is in vicinity of the injection site; the black arrows indicate regions of necrotic muscle tissue (Scale bar = 200 µm). (**B**) High power micrograph of a transverse section of abdominal muscle from the animal displayed in (**A**), arrows indicating regions of muscle necrosis (Scale bar = 10 µm). (**C**) Low power micrograph of a transverse section of the abdominal region of a control group animal 12 h post-injection, displaying healthy muscle with no evidence of necrosis (Scale bar = 200 µm). (**D**) High power micrograph of a transverse section of abdominal muscle from the animal displayed in (**C**) at greater magnification (Scale bar = 10 µm).

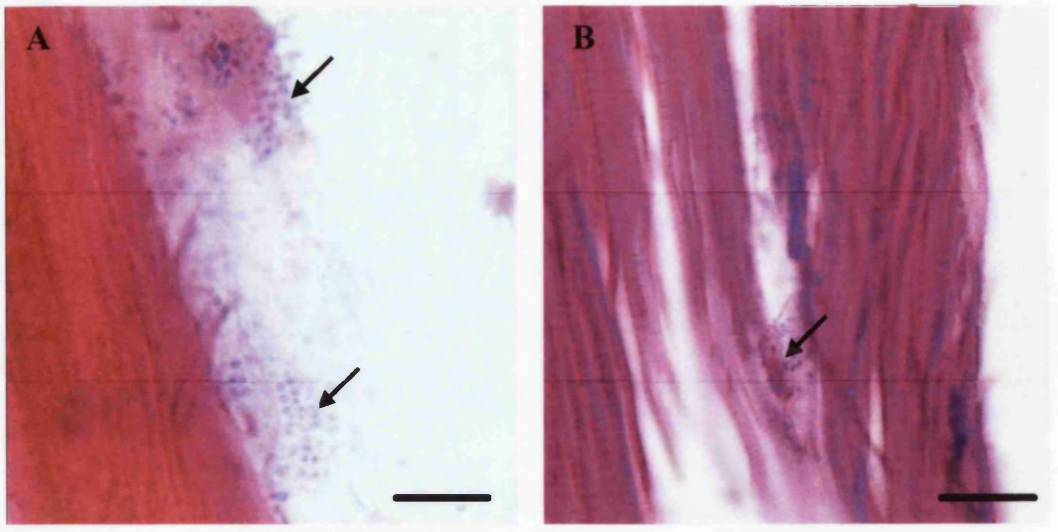


Figure 8. (**A**) High power micrograph of a transverse section of the abdominal region of *Litopenaeus vannamei* displaying muscle fibres and yeast-like organisms denoted by arrows (Scale bar = 10 µm). (**B**) High power micrograph of a transverse section of the abdominal region of *Litopenaeus vannamei* displaying muscle fibres 12 h after receiving the low dose of *V. gazogenes*, arrows indicate position of a cluster of bacteria within the muscle (Scale bar = 10 µm).

4.3.3 Suspect strain *Carnobacterium maltaromaticum* challenge

The suspected *C. maltaromaticum* isolate displayed a higher than anticipated cumulative mortality of 64% when administered at *ca.* 1 x 10^7 bacteria $shrimp^{-1}$ (Figure 9). Despite this, no visible evidence of bacterial infection was found upon histological examination of muscle samples taken from the area around the injection site.

The biofilm samples collected from the tanks containing the suspected *C. maltaromaticum* administered animals were positive for the presence of lactic acid bacteria 24 h after the end of the trial.

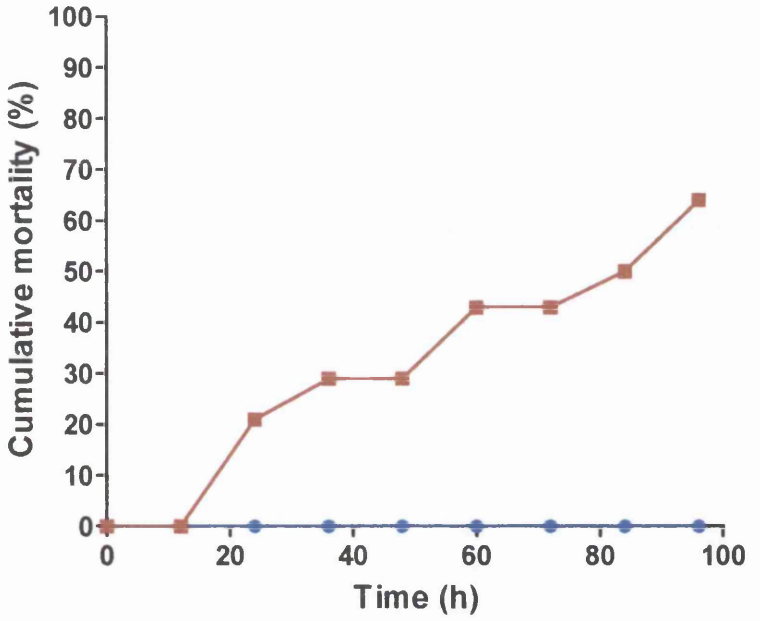


Figure 9. Cumulative mortality of Pacific white shrimp, *Litopenaeus vannamei*, over 96 hr post administration of 1 x 10^7 suspected strain *Carnobacterium maltaromaticum* shrimp^{-1} (---) or 3% NaCl solution control (---).

Table 1. Mean CFU of lactic acid bacteria recovered from *ca.* 50µl of haemolymph obtained from juvenile *Litopenaeus vannamei* administered sham saline or *ca.* 1 x 10^7 cells shrimp^{-1} suspected *C. maltaromaticum*. n=2.

Time (h) post administration	Average CFUs of lactic acid bacteria recovered from haemolymph (per plate)	
	Control (saline)	suspected *C. maltaromaticum* group
24	0	1313
72	0	1536
96	10	460

4.4 Discussion

The fundamental question when selecting a potential probiotic is whether or not it is of benefit to the host organism (Fuller, 1992). Consequently, any candidate micro-organism displaying an unacceptable level of pathogenicity towards the host could not be forwarded as a probiotic and would certainly never gain license for commercial use (Decamp & Moriarty, 2006; Kesarcodi-Watson et al, 2008). Of the three micro-organisms assessed in this chapter, only *Vibrio gazogenes* (NCIMB 2250) displayed sufficiently low lethality when administered intramuscularly to *L. vannamei*, to allow its continuation to the feed trial stage of testing.

Vibrio alginolyticus (NCIMB 1339) exhibited a high level of pathogenicity towards *L. vannamei*, both in terms of cumulative mortality and the rapidity of death. In addition, the estimated LD_{50} value and the presence of moribund animals in the lower dose group, further reinforced the conclusion that this strain was indeed pathogenic to shrimp by injection and therefore probably unsuitable for use as a crustacean probiotic. The estimated LD_{50} value of *ca.* 9×10^6 bacteria shrimp^{-1} determined in this trial was very similar to values obtained for other pathogenic strains of *V. alginolyticus* (Selvin & Lipton, 2003) and *V. campbellii* (Phuoc et al, 2009). The presence of haemocyte nodules within the blood vessels of the gill lamellae is indicative of a haemocyte-mediated immune response to a systemic infection. The lack of any visible muscle damage around the site of injection (when compared to the samples from *V. gazogenes* infected animals) was most likely a result of the speed with which the animals succumbed to the infection (i.e. before any muscle necrosis became apparent).

On initial examination of the data, the suspected *Carnobacterium maltaromaticum* appeared to be the least pathogenic of the three micro-organisms towards *L. vannamei*, with a cumulative mortality of 64% (for a dosage of *ca.* 1 x 10^7 cells shrimp^{-1}). However, the trial of suspected *C. maltaromaticum* was the first conducted and was, in hindsight, ended prematurely. Arguably therefore, in all likelihood the final mortality could have been higher than the recorded 64%. A further factor against the selection of this strain as a potential crustacean probiotic was its persistence within the culture system. It was the only micro-organism of the three tested that was found to have colonised the biofilm of the tanks and filter. In addition, the anti-bacterial activity of suspected *C. maltaromaticum* was considerably less than that observed for the two commercial lactic acid bacteria, *L. plantarum* and *P. acidilactici.* As a consequence, the decision was made not to select the suspected strain *C. maltaromaticum* for feed trial testing in *L. vannamei.* As a footnote, stock of suspected strain *C. maltaromaticum* lost viability shortly after the conclusion of this pathogenicity trial and currently remains un-recovered, despite several attempts to re-isolate from *L. vannamei.*

It should be noted that although *V. gazogenes* displayed pathogenicity towards *L. vannamei* when administered at the higher concentration of *ca.* 3 x 10^7 cells shrimp^{-1}, it remained non-pathogenic at the lower dosage of *ca.* 3 x 10^5 cells shrimp^{-1}. Histopathology of samples from those animals which received the higher dose strongly indicated that the bacteria remained localised in the muscle around the injection site, i.e. the infection was probably not systemic as with *V. alginolyticus.* Given the 'scientific opinion' of the European Commission that micro-organisms utilised in products and feedstuffs should be non-pathogenic, all of the micro-organisms tested should be dismissed as potential crustacean probiotics. However,

given the method of administration and the doses used in these trials there is little likelihood that any micro-organism administered in this fashion would be entirely non-pathogenic toward *L. vannamei.* Consequently, the validity of selecting this method of *in vivo* challenge to test a potential probiotic is debatable. By definition probiotic micro-organisms are administered orally via feed or culture water (Fuller, 1987; Irianto & Austin, 2002). The administration of a high dose of live bacteria directly into a host animal's body via intra-muscular injection is not a realistic simulation of a chance infection and therefore cannot yield a true assessment of the micro-organisms pathogenicity. The scenario is not realistic as it circumvents the crustacean primary defence against parasitic/pathogenic invasion; the cuticle. Consequently, this method of testing takes no account of the primary infectivity of the three micro-organisms, i.e. their ability to gain entry into the host's body, and thus requires the assumption that all strains tested possessed equal and universal infectivity towards the host, which is not the case (Ishibashi & Yamazaki, 2001). Despite this, the activity and lethality of the micro-organisms once present in the host's tissues is still a noteworthy factor in determining their suitability as potential probiotics.

It has recently been noted that species of lactic acid bacteria have been observed associated with human infections and 180 cases of Lactobacillemia and 6 cases of Bifidobacteremia have been recorded over the last 30 years (Sanders et al, 2007). The majority of these incidents involved opportunistic secondary wound infections (Ishibashi & Yamazaki, 2001). However, only two cases of *Lactobacillus* infection in humans have been linked with probiotic consumption and no increase in infection rate has been noted with the increase in probiotic consumption (Sanders et al, 2007). Even though safety assessment is not required by the European Food Safety Authority for

micro-organisms already holding QPS status, in hindsight, the decision not to assess them for pathogenicity towards *L. vannamei* may have been an error.

In conclusion, of the three strains tested only *V. gazogenes* (NCIMB 2250) was selected for further *in vivo* assessment, via oral administration. Despite displaying a degree of pathogenicity towards *L. vannamei* it remained, alongside *L. plantarum* and *P. acidilactici*, the most likely candidate for a novel crustacean probiotic. The fact that *V. gazogenes* did not conform to the European Commission's opinion of a viable micro-organism for use as a probiotic was due more to the testing methods employed. The absence of standardised safety testing criteria for commercially utilised bacteria within Europe (partially relieved by the introduction in 2004 of the European Food Safety Authority's 'Qualified Presumption of Safety' system) should ensure that thorough dietary trials of *V. gazogenes* should be sufficient to demonstrate the micro-organism as safe for use as a crustacean probiotic (Balcázar et al, 2006). However, the degree of pathogenicity displayed by *V. alginolyticus* (NCIMB 1339) coupled with the high number of known pathogenic strains of *V. alginolyticus* (Lee et al, 1996; Selvin & Lipton, 2003; Wang & Chen, 2005) would make it practically impossible to license and market the organism as a crustacean probiotic.

Chapter 5

In vivo assessment of the probiotic potential of *Vibrio gazogenes* and *Lactobacillus plantarum* in the Pacific white shrimp *Litopenaeus vannamei*

Abstract

Selected potential probiotics were assessed with regards to their *in vivo* effects on *Litopenaeus vannamei.* Work undertaken involved the oral administration of *Vibrio gazogenes* and *Lactobacillus plantarum* via feed to post-larval and juvenile shrimp over four feed trials and the subsequent analysis of various physiological parameters. An initial short-term feed trial was conducted to consider the safety of *V. gazogenes* for oral administration to *L. vannamei.* A 28 day trial to assess for any benefit to shrimp growth, survival or feed utilisation conveyed by *L. plantarum* was also performed. These were followed by two longer term trials (8 and 6 weeks, respectively) which examined the effects of the potential probiotics on circulating haemocyte populations, nutritional status and hindgut microfloral diversity. Alongside spread plating, terminal restriction fragment length polymorphism (T-RFLP) analysis was used to detect any alteration in hindgut microbial diversity. The oral administration of potential crustacean probiotics *V. gazogenes* and *L. plantarum* resulted in no discernible enhancement of the health/nutritional status of juvenile *L. vannamei.* In addition, *L. plantarum* administration had no discernible effect on the growth rate, feed conversion rate or survival of post-larval Pacific white shrimp. Animals receiving *V. gazogenes* did, however, show some indications of modulation of the microbial community of the mid and hindguts. The administration of the potential immunostimulant, chitin, also appeared to reduce the levels of *Vibrio*-like organisms in mid/hindgut contents of *L. vannamei.*

5.1 Introduction

Shrimp aquaculture is a massive industry, comprising the bulk of global crustacean aquaculture, with a current annual worth of $50-60 billion (EJF, 2004). In 2001, the UK imported 83,196 tonnes of shrimp worth over £353 million (BBC News website). With the UN FAO estimating that half of the world's seafood demand will need to be met by aquaculture sources by 2020, any means of improving production yields must be investigated (Moriarty, 1999). Currently the shrimp aquaculture industry is focussed in Southeast Asia, where the majority of shrimp farms take the form of open air ponds hacked out of pristine mangroves and wetland habitat. It is estimated that Thailand has lost 83.7% of original mangroves since 1975 (Thornton et al, 2003). If the increased requirement for cultured shrimp is to be met then a shift in culture methods must occur, from open air farms to more economic and environmentally sustainable 'closed' recirculation systems. Such systems would allow greater stocking densities and therefore higher yields while also eliminating the need to relocate farms after only 2-3 years.

With increased stocking densities, however, comes the increased likelihood of serious disease outbreaks particularly in hatchery/nursery facilities. The causative agents in the many disease outbreaks in shrimp aquaculture facilities are bacterial, particularly luminous *Vibrio harveyi* (Moriarty, 1998, 1999). Administration of antibiotics is a highly effective means of pathogen control, but it is far from ideal. In the majority of cases, a mandatory waiting period is required prior to harvesting to allow the antibiotic to clear the animals system, during which the animal may be susceptible to infection. Antibiotics are extremely expensive both to develop and license and with irresponsible use there is the risk of creating antibiotic-resistant strains. No

chemotherapeutic agent is 100% effective and a small proportion of microorganisms will always survive exposure; these may then proliferate and dominate the culture system. If these microbes happen to be shrimp pathogens, then the likelihood of a disease outbreak and subsequent stock loss is exponentially increased.

A different approach must therefore be considered for the control of bacterial pathogens within shrimp aquaculture facilities. Probiotics have been used extensively in agriculture with great success, particularly in intensively reared animals such as chickens and pigs (e.g. Jin et al, 1997; Kyriakis et al, 1999; Patterson & Burkholder, 2003). The idea of administering probiotic bacteria to aquaculture species in an attempt to reduce mortality through disease is not a recent one. Probiotics have several advantages over chemotherapeutic agents such as antibiotics; they can be administered continually (up to harvesting), they are cheap to produce and will not generate resistance in target microorganisims. Probiotics have been extensively tested and commercially utilised in finfish aquaculture for many years (Ringø & Gatesoupe, 1998; Gatesoupe, 1999; Balcázar et al, 2006; Kim & Austin, 2006). Only in recent years, however, has any meaningful research been carried out into the potential of probiotics in invertebrate aquaculture (Rengpipat et al, 2000; Chiu et al, 2007; Castex et al, 2008). The bulk of this research has focused on the effects of probiotic administration on growth parameters, feed utilisation and overall survival (Moriarty, 1998, 1999; Wang, 2007; Farzanfar, 2006).

The work described in this chapter primarily investigates the effects of administration (via feed) of potential probiotics on the diversity/ecology of the hindgut microflora of *L. vannamei*. Any increase in gastrointestinal microfloral diversity triggered would likely benefit the animal by reducing the chances of an opportunistic bacterial pathogen dominating the microflora and thus causing disease. The theory that a

balanced and diverse microflora benefits the host is widely accepted for vertebrates (Patterson & Burkholder, 2003). The increased efficacy and availability of rDNA sequencing techniques such as terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) affords the ability to gain snap shots of the G.I. microfloral ecology of animals receiving probiotics. This is extremely useful as determining the *in vivo* effect of probiotic administration is somewhat problematic. The selection of T-RFLP over DGGE for these experiments was made for purely practical reasons, as the skills and equipment required for T-RFLP were available in the university.

Specific aims of this chapter were:

- The assessment of effects, following the oral administration of *Vibrio gazogenes* and *Lactobacillus plantarum*, on circulating haemocyte populations and nutritional status (hepatosomatic index) in post-larval and juvenile *Litopenaeus vannamei.*

- The examination and analysis of 16S terminal restriction fragment profiles obtained from samples of whole hindgut of *L. vannamei*, for variation in microbial diversity/ecology following administration of potential probiotics *V. gazogenes* and *L. plantarum.*

- The elucidation of any growth/feed utilisation benefits to *L. vannamei* following administration of a commercial feed supplemented with *L. plantarum.*

- The determination of the safety of *V. gazogenes* for oral administration to *L. vannamei.*

5.2 Materials and Methods

5.2.1 Assessment of the safety and effectiveness of selected potential probiotics administered orally via feed to Pacific white shrimp, *Litopenaeus vannamei*

Four feed trials were conducted involving the administration of formulated feeds containing *Lactobacillus plantarum* and *Vibrio gazogenes* (NCIMB 2250) either in isolation, or combination as a multi-species supplement (see Table 1). An initial *V. gazogenes* feed safety trial of 15 days duration was conducted to confirm that the strain was indeed safe for oral administration to *L. vannamei* (Trial 1). The 15 day trial was also utilised for the development and optimisation of sampling protocols employed in the later studies. The safety assessment was then followed by an 8 week feed trial conducted to investigate the effects of orally administered *V. gazogenes* (alongside powdered chitin, utilised as a potential cryo-stabilising agent) on the growth and gut microflora of *L. vannamei* (Trial 3). Also a 28 day trial investigating the effects of orally administered *L. plantarum* on the growth and feed utilisation by post-larval *L. vannamei* was conducted (Trial 2). These were then followed by a 6 week 'multi-species' trial (Trial 4) where feed containing both *V. gazogenes* (plus chitin) and *L. plantarum* was assessed against a 'control' feed containing only *V. gazogenes* (plus chitin).

Table 1. Summary of feed trials conducted.

Trial #	Title	Aims	Duration	Measured parameters
1	An initial 15 day feed trial to assess the safety of orally administered *Vibrio gazogenes* (NCIMB 2250) in *Litopenaeus vannamei*	Assessment of the safety of oral administration of *Vibrio gazogenes* to juvenile *Litopenaeus vannamei* Development and optimisation of sampling protocols/techniques for future feed trials	15 days	Mortality/survival
2	A 28 day *Lactobacillus plantarum* feed trial in *Litopenaeus vannamei*	Assessment of the effect of orally administered *Lactobacillus plantarum* on growth and feed utilisation by post-larval *Litopenaeus vannamei*	28 days	Mortality Average mass gain Feed conversion ratio Specific growth rate
3	An 8 week *Vibrio gazogenes* (NCIMB 2250) feed trial in *Litopenaeus vannamei*	Investigation of the effects of orally administered *Vibrio gazogenes* and potential immunostimulant, chitin; on circulating haemocyte populations, energy reserves and hindgut/faecal microflora of *Litopenaeus vannamei*	56 days	Total & differential haemocyte counts Total & *Vibrio*-like hindgut bacterial load 16S rDNA T-RFLP analysis of whole mid/hindgut contents of *L. vannamei* Hepatosomatic index
4	A 6 week multi-species, *Vibrio gazogenes* (NCIMB 2250) plus *Lactobacillus plantarum* feed trial in *Litopenaeus vannamei*	Investigation of the effects of orally administered *Lactobacillus plantarum*, *Vibrio gazogenes* and potential immunostimulant, chitin; on circulating haemocyte populations, energy reserves and hindgut/faecal microflora of *Litopenaeus vannamei*	42 days	Total & differential haemocyte counts Total & *Vibrio*-like hindgut bacterial load 16S rDNA T-RFLP analysis of whole mid/hindgut contents of *L. vannamei* Hepatosomatic index

5.2.1.1 Trial 1: An initial 15 day feed trial to assess the safety of orally administered *Vibrio gazogenes* (NCIMB 2250) in *Litopenaeus vannamei*

5.2.1.1.a Animals & experimental design

Forty five juvenile *L. vannamei*, 4 ± 0.5 g, were housed in six, 30 l tanks of an open-flow low-volume system at the CSAR facility, Swansea University. The animals were divided into 3 groups each comprising 15 individuals housed over 2 tanks (tanks randomly assigned) with each group receiving a different diet. These diets were a control, consisting of a commercial shrimp maturation feed, a chitin only supplemented diet and a probiotic/chitin supplemented diet. The latter diets comprised the commercial feed top coated with sterile powdered chitin and sterile powdered chitin impregnated with live *V. gazogenes* culture, respectively (details of diet formulation and production are outlined in Section 5.2.1.1.b).

The limited duration of the trial was a result of its primary aim, that of establishing the safety of *V. gazogenes* for oral administration. Animals were fed twice daily with the feed amount equivalent to 5% of tank biomass per day (*ca.* 1.5 g per tank d^{-1}). Prior to feeding, all faeces and uneaten feed were removed and the animals examined *in situ* for signs of distress/ill health.

After 15 successive days of feeding the trial was ended and the animals sacrificed (euthanised on ice) and utilised in the development of the sampling methodologies employed in the following longer term feed trials. The optimisation of these techniques required numerous modifications and unfortunately due to this, no data were obtained for the animals in this trial aside from a record of survival/mortality.

5.2.1.1.b Preparation of feed

As stated in Section 5.2.1.1.a, the control group diet used was a commercial shrimp maturation pelleted feed (Dragon Feeds Supreme™) supplied by Dragon Feeds Ltd., Port Talbot, UK. This feed was also the main constituent of the two experimental diets administered. The probiotic group was fed a diet of Dragon Feeds Supreme™ top coated with chitin powder (Sigma-Aldrich; Cat# 417955-1KG) impregnated with live *V. gazogenes* culture. *V. gazogenes* was cultured in TSB (plus 2% NaCl) at 25°C for 24 h, prior to centrifuging (3255 x g, 12 min, RT). The pellet was retained and its weight determined before being mixed, aseptically, with an equal quantity of sterile powdered chitin. The function of chitin was to act as a potential cryo-protectant during drying and storage of the feed, as a characteristic of the Vibrionaceae is an ability to bind chitin and research indicates that *Vibrio* spp. can tolerate greater variations in environmental parameters, such as temperature, when associated with chitin (Nalin et al, 1979; Amako et al, 1987). A powdered (insoluble), rather than a soluble form of chitin was selected as the chitin/bacteria was required to remain associated with the feed in order to be ingested by the animals. The mixture was left in a covered 90 mm Petri dish for 24 - 48 h at 25°C to allow for the adhesion of the bacteria to the chitin particles. The final feed formulation contained a mass of the probiotic/chitin mixture equivalent to 1% of the total (final) mass of feed. The probiotic/chitin mix was added to sunflower oil (Tesco Value; volume equivalent to 5% of the final mass of feed) and manually homogenised until a universally consistent suspension was obtained. This suspension was then added to a quantity of Dragon Feeds Supreme™ equivalent to 94% of the required mass of finished feed and mixed by hand until consistent. The feed was then spread onto foil and allowed to air dry at

RT for 24 – 48 h, before being stored at 4°C. The feed administered to the chitin only diet group was produced as a stated above, but with sterile 3% NaCl solution in place of the spun down *V. gazogenes* culture material.

The viability of the probiotic/chitin mixture was elucidated via standard dilution and spread plating techniques as containing *ca.* 3 x 10^9 CFU g^{-1} (equating to an estimate of *ca.* 3 x 10^7 cells g^{-1} of feed). The latter is merely an estimate, however, as no viable CFU could be recovered from the final feed.

5.2.1.2 Trial 2: A 28 day *Lactobacillus plantarum* feed trial in *Litopenaeus vannamei*

5.2.1.2.a Animals & experimental design

Two diet groups each containing 54 post-larval *L. vannamei* (0.5 ± 0.05 g) were utilised to assess the short term effects of the oral administration of *L. plantarum* on growth, survival and feed utilisation (feed conversion ratio; FCR) in Pacific white shrimp. Each diet group was housed over three randomly assigned, 30 l tanks (18 animals per tank) in an open flow low volume system at the CSAR facility, Swansea University.

The first group was fed an un-supplemented diet and designated the control diet group; the remaining animals received a diet top-coated with *L. plantarum* culture and were designated the probiotic diet group (details of diet formulation and production are outlined in Section 5.2.1.2.b). The animals were fed twice daily with the feed amount equivalent to 11% of tank biomass per day (*ca.* 1 g per tank d^{-1}). Prior to

feeding, all faeces and uneaten feed was removed from the tanks, the unconsumed feed was collected separately from the faeces, dried at RT and weighed. Five randomly selected animals from each tank were weighed weekly; these values were used to estimate the biomass of each tank and thus the amount of feed required for the subsequent 7 days. Accurate tank biomass determinations were made, via the weighing of all animals, at the start of the trial, after 14 days of feeding and at the end of the trial (28 days). From these data average values for each tank for; individual animal weight, weight gain per day, percentage growth and specific growth rate (SGR), were determined. In addition, the biomass values along with the mass of feed consumed (calculated by subtracting the amount of un-consumed feed from the amount administered) were used to estimate the feed conversion ratio (FCR). Percentage survival was also recorded for each group.

5.2.1.2.b Preparation of feed

As for Trial 1 (see Section 5.2.1.1) a commercial shrimp maturation diet (Dragon Feeds Supreme™) was utilised as the basis for the supplemented diets. The control and *L. plantarum* diets were produced using the same ratio of constituents and method of top-coating outlined in Section 5.2.1.1.b. In the case of the *L. plantarum* supplemented diet, the *V. gazogenes* culture/chitin mix was replaced by a powdered mix of lyophilised *L. plantarum* culture and skimmed milk powder (with the latter performing the role of cryo-protectant). The lyophilised powder was supplied by the project's industrial sponsor, Cultech Ltd. Baglan, UK and contained *L. plantarum* at a concentration of *ca.* 1.6×10^{11} CFU g^{-1}. The viability of the final feed was estimated via standard dilution and spread plating techniques as *ca.* 2×10^{8} CFU g^{-1}.

The control feed was produced as described in Section 5.2.1.1.b, but with the chitin powder (plus 3% NaCl solution) replaced by skimmed milk powder (also obtained from Cultech Ltd.). All feed was stored in sealed containers at 4°C until required, in order to preserve maximum viability of the probiotic. Fresh feed was produced every 14 days.

5.2.1.3 Trial 3: An 8 week *Vibrio gazogenes* (NCIMB 2250) feed trial in *Litopenaeus vannamei*

5.2.1.3.a Animals & experimental design

The experimental design utilised in the 8 week feed trial was based largely on that used for Trial 1 (Section 5.2.1.1.a) but expanded in scope. Whereas the aim of Trial 1 was merely to assess the survival and mortality of *L. vannamei* when fed *V. gazogenes*, the principal purpose of this third trial was to investigate the effects of *V. gazogenes* administration on the mid/hindgut microflora of *L. vannamei*, using microbiological and molecular biology techniques. In addition, the nutritional status of the diet groups were assessed via determination of the hepatosomatic index (HSI). Haemocyte counts were also performed to assess the effect of *V. gazogenes* administration on circulating haemocyte populations. Three diet groups were established, as for Trial 1 these were a control diet group, a chitin (only) supplemented diet group and a *V. gazogenes*/chitin supplemented diet group. Forty animals (juvenile *L. vannamei*; 10.5 ± 2.5 g) were allocated to each diet group at the start of the trial (120 shrimp total); these animals were housed over twelve, 25 l tanks

of a closed, recirculation system at the CSAR Facility, Swansea University. Only 30 animals were required for sampling per group, however, given the duration of the trial it was thought prudent to maintain a surplus of animals in the event of mortality. The commencement of feeding (i.e. trial start time, T0) was staggered for each diet group, by 2 days, this allowed sufficient sampling time whilst maintaining comparability between the diet groups results.

The animals were fed twice daily with the feed amount equivalent to 4% of the tank biomass per day (*ca.* 4 g per tank d^{-1} for the first week). Ten randomly selected animals from each diet group were weighed weekly to gain an estimate of the average biomass per tank and therefore the amount of feed required for the following seven days. Prior to feeding, faeces, uneaten feed and any mortalities were removed from the tanks. In addition to this, the tank filters were cleaned and 50% water exchanges performed daily, to maintain water quality.

Fifteen animals were sacrificed for baseline samples at the commencement of the trial (Section 5.2.1.3.c to 5.2.1.3.e); these animals were not members of the diet groups, but were collected from the CSAR main facility at the same time as the trial animals. Fifteen animals from each diet group (selected at random over the four tanks) were sacrificed and sampled as detailed in Section 5.2.1.3.c-e, 4 weeks into the trial. The selected animals were removed from the tanks 3 h after receiving their morning feed. The remaining animals in each diet group were then combined and redistributed over a suitable number of tanks (*ca.* 10 animals per tank) to minimise variation due to reduced stocking densities. At the end point of the trial (after 8 weeks of feeding) a further 15 animals from each diet group, again randomly selected from the remaining tanks, were sacrificed and sampled.

5.2.1.3.b Preparation of feed

The control diet used was a commercial shrimp maturation feed (Dragon Feeds Supreme™) obtained from Dragon Feeds Ltd., Port Talbot, UK. The chitin only and the chitin plus *V. gazogenes* culture feeds were produced and stored as described in Section 5.2.1.1.b.

5.2.1.3.c Haemocyte counts

As stated in Section 5.2.1.3.a sampling of the three diet groups was staggered and separated by a 2 day interval. The control diet group was sampled first, followed by the chitin diet group and finally the *V. gazogenes*/chitin diet group. Sampling was, however, carried out at the same time of day.

After weighing and numbering, 5 (of the fifteen) animals were partially anaesthetised on ice for *ca.* 3 min to minimise movement. Haemolymph was drawn into a 2 ml syringe containing 500 µl of sterile, ice cold shrimp MAC (Appendix 1; Chiu et al, 2007). The animals were bled from the main ventral vessel at the second/third abdominal segment, using a sterile 21 gauge needle. The volume of haemolymph extracted was recorded for use in calculating the dilution factor (require for determining the total haemocyte count (THC)). Five hundred microlitres of the contents of the syringe were then transferred to a 1.5 ml Eppendorf tube containing 500 µl of isosmotic formalin solution (Appendix 1) and used to perform the differential haemocyte counts (DHC). The remaining contents of the syringe were then transferred to a second sterile 1.5 ml Eppendorf tube and placed on ice until the remainder of the samples were collected. Twenty five microlitres of the

haemolymph/MAC suspension was pipetted onto a Neubauer haemocytometer and cell counts performed under x40 magnification, using a Leitz Wetzlar light microscope.

Differential haemocyte counts were performed on slides prepared using a Shandon cytocentrifuge. One hundred microlitres of the formalin fixed haemolymph/MAC suspension was centrifuged (1000 rpm; 5 min) and the slides allowed to dry at room temperature, before being fixed again in absolute methanol and stained using undiluted Wright's stain (~ 2 min). Once dry, the numbers and proportions of each haemocyte type were determined using a minimum of 200 cells per slide. Cell types were identified using morphological criteria modified from that outlined by Bauchau (1981).

5.2.1.3.d Mid/hindgut and faecal microbiology

The following methods were utilised to determine the total number of aerobic colony forming units and the number of colony forming units of *Vibrio*-like bacteria present in the faeces and mid/hindgut contents of *L. vannamei*. The function of this was to investigate whether the administration of *V. gazogenes* and/or chitin affected the total number of bacteria and/or the relative proportion of *Vibrio*-like organisms in the mid/hindgut.

The 5 animals from which haemolymph samples were collected in Section 5.2.1.3.c were euthanised on ice (*ca.* 10 min) and their mid/hindguts aseptically removed. The mid/hindguts were stripped of faeces and discarded; the faecal material collected from each animal was then transferred (aseptically) to sterile 1.5 ml Eppendorf tubes. The mass of faeces was determined by subtracting the mass of the empty tube from its

mass when full, the tubes having been numbered and weighed prior to sampling. One millilitre of sterile 3% NaCl solution was then added to each tube and their contents mixed by a combination of manual homogenisation and vortexing (*ca.* 1 min). A x2,000 dilution was performed and 100 µl of the dilutant aseptically spread onto a TSA (plus 2% NaCl) plate and duplicate plates were produced for each sample. The plates were then incubated aerobically at 27°C for 24 h and colony counts performed. The number of colony forming units per gram of faeces (wet weight) was then calculated for each animal. For an estimate of the number of colony forming units of *Vibrio*-like bacteria per gram of faeces, 100 µl of undiluted faecal suspension was aseptically spread onto plates of thiosulfate citrate bile salts sucrose agar (TCBS agar) (BD-Difco; Cat# 265020). TCBS agar is regarded as being specific for *Vibrio*-like species of bacteria (Castex et al, 2008). The TCBS plates were also incubated aerobically at 27°C for 24 h after which colony counts were performed.

5.2.1.3.e Hepatosomatic index and mid/hindgut sampling for molecular analysis

The remaining 10 animals were weighed and numbered before being euthanised on ice (*ca.* 10 min). Mid/hindgut and whole hepatopancreas were aseptically removed; the mid/hindgut was stripped of faeces which was collected and weighed as described in Section 5.2.1.3.d before being stored, at -20°C, for later molecular analysis (Section 5.2.1.5) This method was modified for the week 8 sampling, where the whole mid/hindgut (plus faeces) was used rather the faeces alone; this modification was incorporated into the sampling for all subsequent trials. The wet weight of hepatopancreas was determined and along with the whole shrimp weight used to

calculate the animal's hepatosomatic index (HSI), via the method used by Castex et al. (2008).

5.2.1.3.f Statistical analysis

To determine any significant differences between the diet groups an ANOVA together with a Bonferroni multiple comparisons post test was used. This followed the determination of normal distribution of the data via the application of a Kolmogorov-Smirnov test. In cases where the standard deviation of the data was not equal between groups, a Kruskal-Wallis test (nonparametric ANOVA) with a Dunn's multiple comparison post test was utilised. All values are shown as arithmetic means ± 1 standard error of the mean (S.E.M).

5.2.1.4 Trial 4: A 6 week multi-species, *Vibrio gazogenes* (NCIMB 2250) plus *Lactobacillus plantarum* feed trial in *Litopenaeus vannamei*

5.2.1.4.a Animals & experimental design

Two diet groups were utilised for this trial; a control group, fed a diet supplemented with the *V. gazogenes*/chitin mix and a multi-species group receiving a diet containing both the *V. gazogenes*/chitin mix and lyophilised *L. plantarum*. Each diet group contained 35 animals (10 ± 2 g), housed over three, 25 l tanks of a closed, recirculation system at the CSAR facility, Swansea University. All feeding procedures and animal husbandry tasks were conducted as outlined in Section 5.2.1.3.a; sampling

and statistical analysis was conducted using the methods described in Sections 5.2.1.3.c-f. Molecular analysis of faecal and mid/hindgut samples was performed as described in Section 5.2.1.5. As for Trial 3, the commencement of feeding (i.e. trial start time, T0) was staggered for each diet group, by 2 days.

Baseline (T0) samples were obtained from 10 additional animals collected from the CSAR main culture system at the commencement of the trial. The intention was to sample 15 animals from each group after 3 and 6 weeks of continuous feeding, however, both diet groups experienced unexpectedly high mortality due to issues associated with culture water quality, consequently only the 6 week sampling could be performed.

5.2.1.4.b Preparation of feed

Immediately prior to this trial the opportunity arose to formulate and produce pellet feed containing the required probiotic supplements rather than simply top-coating a commercial feed. The principle benefits of this were the homogeneity of the supplements within the feeds and their guaranteed consumption by the animals.

The base composition of the feeds was as follows; (required to produce *ca.* 1 kg of feed) 260 g fish meal 66, 260 g polychaete meal, 100 g wheat gluten, 260 g wheat starch, 10 g vitamin mix, 0.8 g Stay C powder (stabilised vitamin C), 20 g $CaSO_4$, 20 g alginate, 20 g dicalcium phosphate powder, 50 g fish oil, 20 g of probiotic supplement/s and *ca.* 300 ml of water. In the case of the control feed the 20 g per kg of supplement was composed of 10 g skimmed milk powder and 10 g of the *V. gazogenes*/chitin mix (produced as outlined in Section 5.2.1.1.b). The multi-species

feed contained 10 g per kg of the *V. gazogenes*/chitin mix and an equal mass of the lyophilised *L. plantarum* powder, described in Section 5.2.1.2.b.

Production of the feed involved the weighing and thorough mixing of the dry components, both by hand and electric whisk. This was followed by the addition of the fish oil and sufficient water to render the mix, dough-like. Water was added in small volumes whilst mixing in order to achieve the required consistency for milling. The feed was then milled and extruded using an E12 Meat mincer (Bertolini Spa, Reggio Emila, Italy) fitted with a 3 mm mincer plate and placed on trays in preparation for drying. The feed was then placed in a 200 litre drying oven (GenLab Ltd. Widnes, UK; Model #OV/200/F) at 45°C for *ca.* 36 h. Once dry, the strands of feed were placed in a kitchen food processor and processed until pellets of *ca.* 5 mm length were obtained, the feeds were then stored at 4°C until required.

The viability of the feeds was determined through the dilution and spread plating of homogenised samples. *L. plantarum* was present at a concentration of 2×10^6 CFU g^{-1} of the multi-species diet, *V. gazogenes*, however, was unrecoverable from either feed. The *V. gazogenes*/chitin mix originally contained 1×10^8 CFU g^{-1}, therefore a cell concentration of 1×10^6 cells g^{-1} can be inferred for both diets.

5.2.1.5 Molecular analysis of mid/hindgut and faecal samples obtained from *L. vannamei*

The molecular technique employed in the analysis of the microbial ecology of faeces or whole mid/hindgut of animals sampled in the feed trials described in Sections 5.2.1.3 & 5.2.1.4, was terminal restriction fragment length polymorphism (T-RFLP). The methodology used was slightly modified from that described by Gregory (2008) and is detailed in Sections 5.2.1.5.a-f.

5.2.1.5.a DNA extraction

Samples of whole mid/hindgut and/or faeces obtained as outlined in Sections 5.2.1.3 & 5.2.1.4 were paired and pooled prior to extraction. Therefore, the 10 animals sampled from each diet group at each sampling period, yielded 5 distinct samples for DNA analysis. The samples were pooled in this fashion to ensure that a sufficient quantity of microbial DNA was present for extraction and amplification.

The pooled samples were then manually homogenised for *ca.* 1 min before extracting the DNA using a fastDNA SPIN kit for soil (Q-Biogene, Cambridge, UK). Post homogenisation, the sample was added to a lysing matrix tube, followed by 980 µl of sodium phosphate buffer and 120 µl MT buffer. The lysing matrix tubes were then placed in a FastPrep® Instrument and processed at speed setting five for 2 x 30 sec. The tubes were then centrifuged (13,000 rpm; 5 min at RT). The supernatant was then transferred to a sterile 1.5 ml Eppendorf tube and 250 µl of PPS (protein precipitate solution) was added. The contents were then gently mixed by inverting the tubes 10 times, by hand. The tubes were centrifuged (13,000 rpm; 1 min at RT) before the

supernatant was transferred to a sterile 15 ml tube containing 1 ml of binding matrix suspension. After 2 min of gentle mixing, the silica matrix in the tubes was allowed to settle for *ca.* 3 min. At this point, 500 µl of the supernatant was carefully removed and discarded. The remaining binding matrix was resuspended and transferred to a SPIN™ filter it was then centrifuged (13,000 rpm; 1 min at RT) to capture the matrix-bound DNA. The bound DNA was then washed (13,000 rpm; 1 min at RT) with 500 µl of SEWS-M (salt/ethanol wash solution; DNase-free). An additional centrifugation was performed to remove any remaining SEWS-M from the filter before eluting the DNA into a fresh catch tube using 50 µl ddH_2O and centrifuging for 1 min at 13,000 rpm. The samples were stored at -20°C until required, DNA quantifications were carried out using a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Products, Wilmington, USA).

5.2.1.5.b Polymerase chain reaction

DNA (0.5 µl) was added to a PCR mix comprising 1xGoTaq® flexi buffer, 1.25U GoTaq® Hot Start Polymerase, 200 µM each dNTP, 0.25 µM each primer, 1.25 mM $MgCl_2$ (all PCR mix components supplied by Promega UK, Southampton, UK). The forward primer for PCR reactions was labelled with a Beckman D3 dye (Sigma-Aldrich Genosys). Details of the primers used can be found in Table 2.

Polymerase chain reactions were performed in triplicate using a PTC-200 DNA Engine (M.J. Research Inc., Waltham, USA). The three PCR products produced from each sample were pooled prior to analysis to minimise PCR amplification bias. A no template reaction (negative control) with DNA replaced by ddH_2O was always carried out.

Table 2. Universal eubacterial primers targeting 16S rDNA.

Gene	Primer name	Sequence	Reference
16S rDNA	WellRED D3 labelled 27f	5'[D3]-AGA GTT TGA TCM TGG CTC AG-3'	(Lane, 1991)
16S rDNA	1387r	5'-GGG CGG WGT GTA CAA GGC-3'	(Marchesi et al., 1998)

16S rDNA PCR program:

- Initial denaturation at 95°C for 5 min followed by 30 cycles of;
  - Denaturation (95°C for 30 sec)
  - Annealing (65°C for 45 sec)
  - Extension (72°C for 75 sec)
- Final extension step of 72°C for 10 min.

5.2.1.5.c Agarose gel electrophoresis

The PCR products were checked visually using gel electrophoresis; 0.8 % w/v gels were used to visualise the 16S rDNA. Fifty or 100 ml gels were produced by the addition of the appropriate mass of agarose powder (Fisher Scientific UK Ltd., Loughborough, UK) to 1xTBE electrophoresis buffer (Appendix 1). Half a microgram ml^{-1} of ethidium bromide was added after melting the agarose in a microwave. Three microlitres of PCR buffer was mixed with 1 µl bromophenol blue loading dye (Fisher Scientific UK Ltd.) and run for *ca.* 30 min at 100 volts. DNA was visualised using a Bio-Rad UV transilluminator (Bio-Rad Laboratories Ltd., Hemel. Hempstead, UK) at 245 nm.

5.2.1.5.d Restriction endonuclease digestions

Standard enzyme digests were carried out over a duration of five hours, or overnight, at 37°C using the following mixture; 15 µl sample DNA, 5 µl digestion buffer, 1 µl of *Hph*1 enzyme (New England Biolabs, USA) and 34 µl ddH_2O.

5.2.1.5.e PCR product purification

A QIAquick™ PCR purification kit (Qiagen Ltd., Crawley, UK) was used to remove excess primers from the PCR product. PCR samples were mixed with five times the volume of binding buffer PB, before the mixture was added to a QIAquick™ spin column. The column was then centrifuged (13,000 rpm; 1 min at RT) in order to bind the DNA to the silica membrane. The flow-through was discarded and the DNA washed via further centrifugation (13,000 rpm; 1 min at RT) with 750 µl of wash buffer PE. The flow-through was again discarded and the column further centrifuged (as previously) to remove any remaining wash buffer. The DNA was eluted via the addition of 30 µl DNase/pyrogen free water to the centre of the membrane. The column was allowed to stand for 1 minute before being transferred to a fresh, sterile 1.5 ml Eppendorf tube and centrifuged (13,000 rpm; 1 min at RT). The eluted DNA sample was then ready for fragment analysis.

5.2.1.5.f Fragment analysis

Three microlitres of the purified DNA eluant was mixed with 37 µl of sample loading solution (Beckman Coulter UK Ltd., High Wycombe, UK). Fragment analysis was

performed using Beckman CEQ 8000 capillary electrophoresis, utilising a 640 base pair standard (Beckman Coulter UK Ltd.). Analysis was performed using a capillary temperature of 50°C and the following program settings;

- Denature: 120 sec
- Injection: 2 kV for 45 sec
- 1° separation: 5 kV ramp duration 2 min
- 2° separation: 5 kV, start time 10 min, ramp duration 5 min
- Total separation time: 75 min

In order to minimise the false reporting of background noise, the cut-off level for peak recognition was set at 5 percent. This method of fragment analysis is known to produce "shoulder peaks" around the main product, to eliminate these T-RFLP profiles were edited by eye. The moving average function of the software program T-Align (Smith et al., 2005) was then utilised in the identification of common fragments. The resultant fragment groupings were then also edited by eye to minimise the miss-grouping of peaks. Multivariate statistical package (MVSP) (Kovach Computing Services, Anglesey, UK) was used to conduct principal component analysis on the T-RFLP data set. However, the T-RFLP relative abundance data was first transformed using a chord transformation (Ramette, 2007), to make it both suitable for linear PCA analysis and to avoid over representation of changes in rare terminal restriction fragments. The scores of the first two principal components were used to determine any differences between the T-RFLP sample profiles of the various diet groups.

5.3 Results

5.3.1 Trial 1: An initial 15 day feed trial to assess the safety of orally administered *Vibrio gazogenes* (NCIMB 2250) in *Litopenaeus vannamei*

No mortality was recorded in any of the three diet groups during the 15 days of this feed safety trial. Despite the lack of useful data, the sampling performed at the end of this trial was regarded as successful as it allowed the development and optimisation of the techniques described in Sections 5.2.1.3.c-e.

5.3.2 Trial 2: A 28 day *Lactobacillus plantarum* feed trial in *Litopenaeus vannamei*

No statistically significant differences were observed between the control and *L. plantarum* supplemented diet groups for any of the feed utilisation or growth parameters assessed ($P<0.05$) (Table 3). Although the *L. plantarum* supplemented group exhibited greater mortality over the course of the trial, compared to the control group (Figure 1) this difference was also not statistically significant ($P>0.05$).

Table 3. Feed utilisation and growth data obtained for post-larval *Litopenaeus vannamei* fed a commercial shrimp maturation diet and the equivalent diet supplemented with the probiotic bacterium, *Lactobacillus plantarum*, for 28 days. Values displayed as mean ± SEM.

Diet Group	Average initial animal mass (g)	Average final animal mass (g)	Average mass gain per animal over trial (g)	Average mass of feed consumed per animal (g)	Feed Conversion Ratio	Specific Growth Rate (%)
Control	0.49 ± 0.03	1.63 ± 0.06	1.13 ± 0.03	2.00 ± 0.07	1.77 ± 0.09	4.27 ± 0.18
L. plantarum supplemented	0.50 ± 0.04	1.66 ± 0.08	1.15 ± 0.05	2.14 ± 0.07	1.86 ± 0.08	4.26 ± 0.26

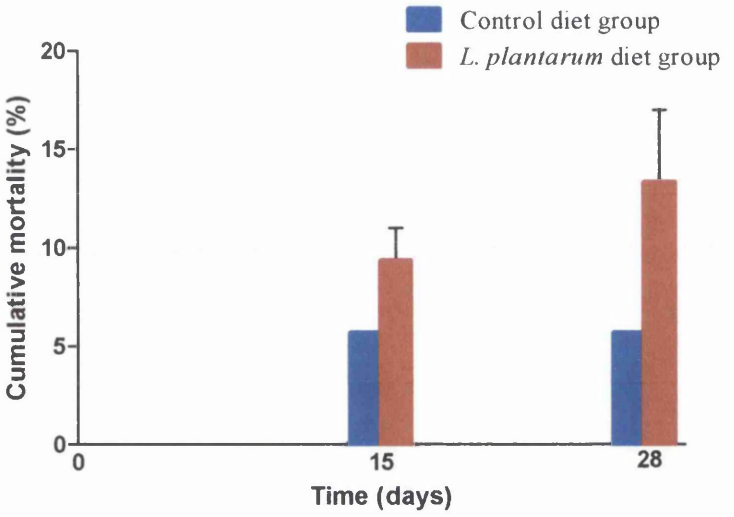


Figure 1. Cumulative mortality of post-larval Pacific white shrimp, *Litopenaeus vannamei*, over 28 day feed trial assessing effects of *Lactobacillus plantarum* administration on feed utilisation and growth parameters. Mean values ± SEM, n = 3.

5.3.3 Trial 3: An 8 week *Vibrio gazogenes* (NCIMB 2250) feed trial in *Litopenaeus vannamei*

5.3.3.a Haemocyte counts

Statistically significant differences were observed among the total and differential haemocyte counts obtained for the baseline, control and experimental diet groups over the 8 week feeding period (Figures 2-5). The total number of control group haemocytes, more specifically, the number of control granulocyte-type haemocytes and hyaline cells at week 4 were significantly higher than the corresponding baseline values (Figures 2 & 3). At week 8, however, there were no statistically significant differences in total circulating haemocyte numbers and granulocyte-type cell numbers between the baseline data and the three diet groups. The only statistically significant variation displayed by the chitin diet group occurred in the week 4 data for granulocyte-type cell numbers (Figure 3). The week 8 counts for the *V. gazogenes*

plus chitin diet group semi-granulocyte and hyaline cells were significantly higher than those of the baseline and corresponding control (Figures 4 & 5).

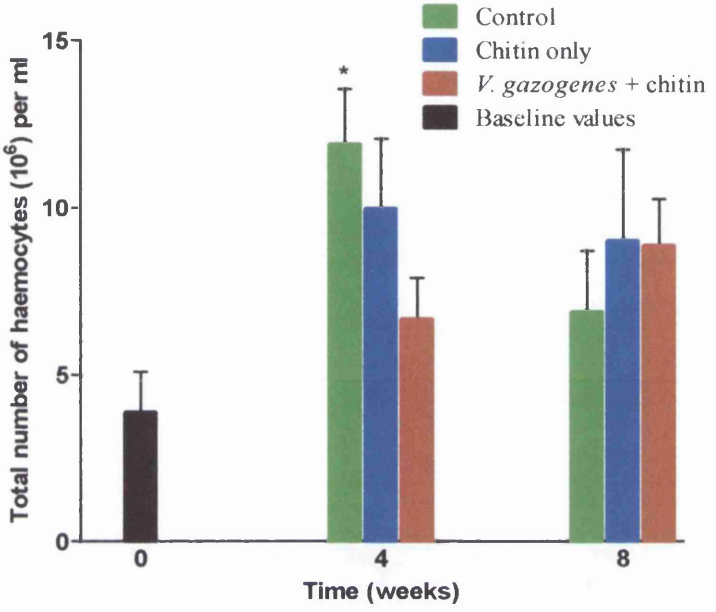


Figure 2. Total haemocyte numbers observed in haemolymph samples from baseline animals as well as those from the control, chitin only and chitin plus *Vibrio gazogenes* diet groups at 4 week intervals over an 8 week feeding period. Mean ± SEM, n=5, * P<0.05 compared with baseline.

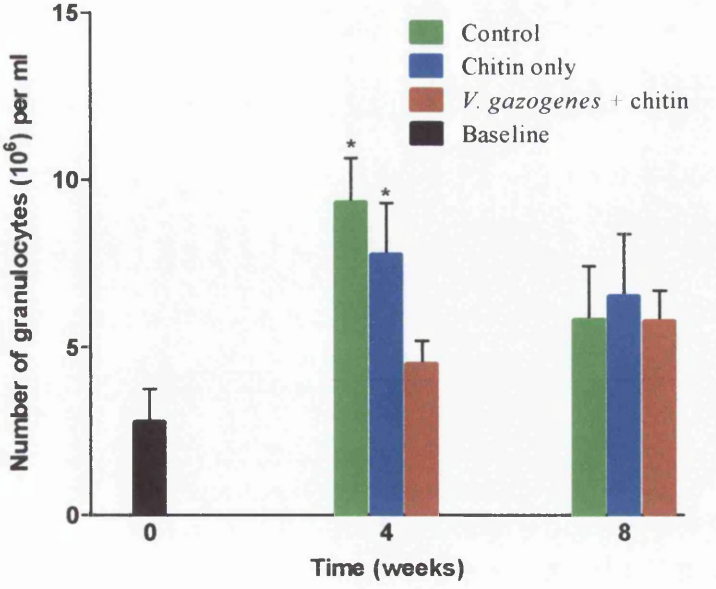


Figure 3. Number of granulocyte type haemocytes observed in haemolymph samples from baseline animals as well as those from the control, chitin only and chitin plus *Vibrio gazogenes* diet groups at 4 week intervals over an 8 week feeding period. Mean ± SEM, n=5, * P<0.05 compared with baseline.

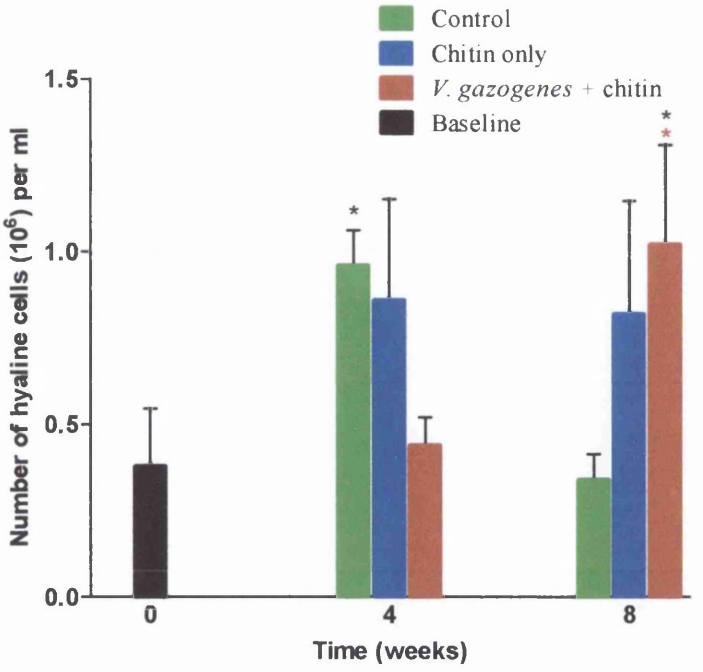


Figure 4. Number of hyaline type haemocytes observed in haemolymph samples from baseline animals as well as those from the control, chitin only and chitin plus *Vibrio gazogenes* diet groups at 4 week intervals over an 8 week feeding period. Mean ± SEM, n=5, * P<0.05 compared with baseline; * P<0.05 compared with control at same time period.

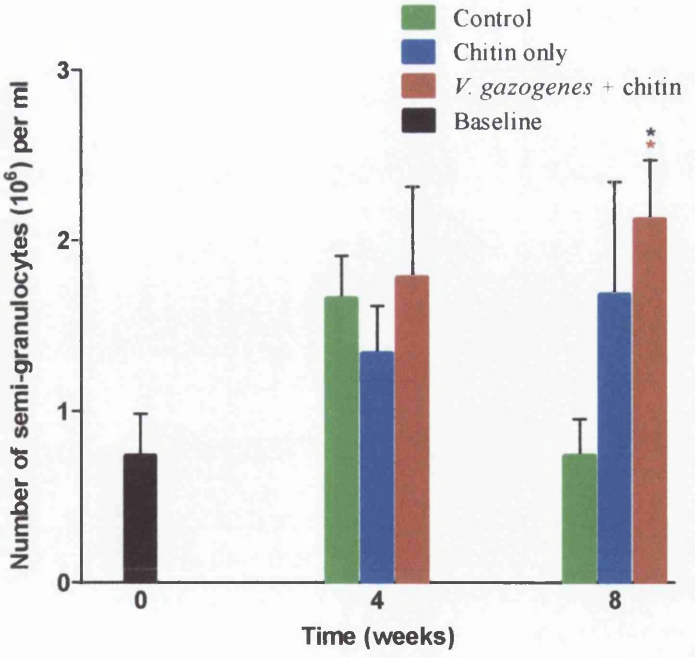


Figure 5. Number of semigranulocyte type haemocytes observed in haemolymph samples from baseline animals as well as those from the control, chitin only and chitin plus *Vibrio gazogenes* diet groups at 4 week intervals over an 8 week feeding period. Mean ± SEM, n=5, * P<0.05 compared with baseline; * P<0.05 compared with control at same time period.

5.3.3.b Mid/hindgut content microbiology

As can be seen from Table 3 there was a great deal of variation in both total and *Vibrio*-like counts in the baseline samples. For example total bacterial counts ranged from 0.93 – 22.89 x 10^7 g^{-1}. Analysis of the colony forming unit data obtained for *Vibrio*-like mid/hindgut bacteria indicated statistically significant differences between the chitin and *V. gazogenes* plus chitin diet groups and those of the baseline values (Table 3). At week 8, the samples from both groups receiving chitin supplemented diets exhibited a significantly reduced proportion of bacteria displaying *Vibrio*-like growth characteristics compared to the baseline (Table 3). As well as this, the chitin only group exhibited a statistically significant difference compared to the corresponding control samples (in week 8). In addition, at week 4, the diet group receiving *V. gazogenes* also displayed significantly reduced *Vibrio*-like bacteria compared to control group samples.

Table 3. Total aerobic bacterial and aerobic *Vibrio*-like bacterial colony forming units present in samples obtained from the mid/hindgut of *Litopenaeus vannamei* receiving control, chitin or chitin plus *Vibrio gazogenes* diets. (Mean ± SEM, n=5, * $P<0.05$ compared with baseline; * $P<0.05$ compared with appropriate control at same time period).

Diet type administered	Period of feed administration	Total bacterial content of hindgut (CFU g^{-1} wet weight)	*Vibrio* -like bacterial content of hindgut (CFU g^{-1} wet weight)	% of total culturable bacteria with *Vibrio* -like growth characteristics
Control†	Baseline (0 wk)	6.78 ± 2.38 x 10^7	5.73 ± 3.60 x 10^6	4.3 ± 1.5
Control†	4 weeks	3.77 ± 0.76 x 10^7	0.66 ± 0.29 x 10^6	2.4 ± 1.3
Chitin	4 weeks	3.06 ± 0.66 x 10^7	0.40 ± 0.17 x 10^6	1.5 ± 0.6
V. gazogenes plus chitin	4 weeks	2.28 ± 0.31 x 10^7	0.11 ± 0.05 x 10^{6**}	0.5 ± 0.2 **
Control†	8 weeks	2.25 ± 0.57 x 10^7	0.38 ± 0.14 x 10^6	3.9 ± 2.5
Chitin	8 weeks	2.99 ± 0.36 x 10^7	0.13 ± 0.05 x 10^{6*}	0.5 ± 0.1 **
V. gazogenes plus chitin	8 weeks	5.15 ± 0.77 x 10^{7*}	0.42 ± 0.08 x 10^6	0.7 ± 0.1 *

†Dragon Feeds Supreme™.

5.3.3.c Hepatosomatic Index

A statistically significant difference was observed between the hepatosomatic index values obtained for the control diet group (at week 4) and those of the baseline samples (Figure 6). A significant difference was also noted between the week 4 samples of the control diet group and those of the chitin only and the chitin plus *V. gazogenes* diet groups (Figure 6). However, no such variations were evident in the sample data gathered during week 8.

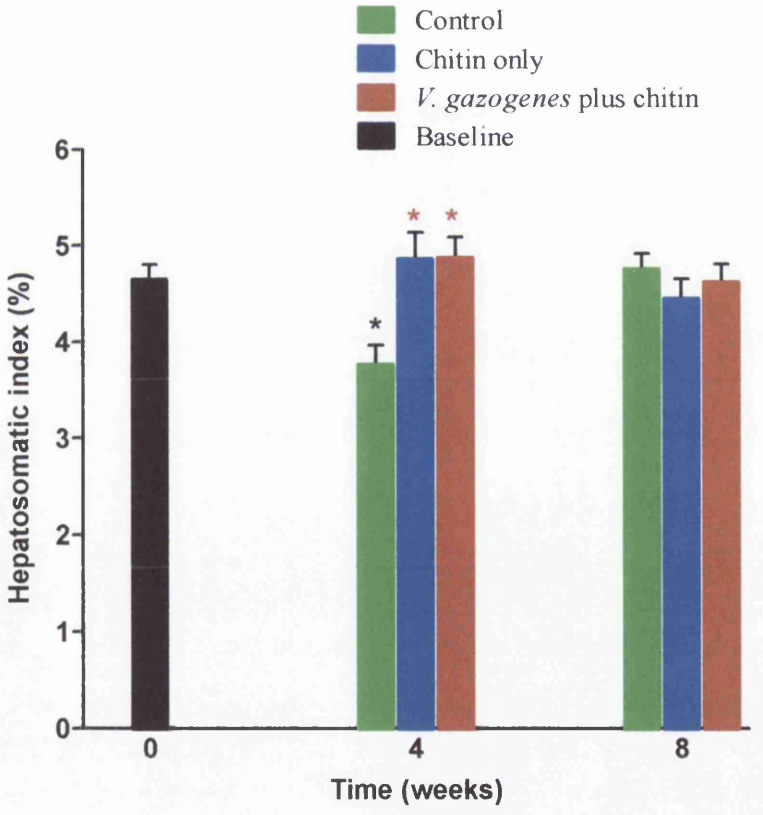


Figure 6. Hepatosomatic index of baseline animals as well as those of the control, chitin only and chitin plus *Vibrio gazogenes* diet groups at 4 week intervals over the 8 week feeding period. Mean ± SEM, n=10, * $P<0.05$ compared with baseline; * $P<0.05$ compared with appropriate control at same time period.

5.3.3.d 16S rDNA T-RFLP analysis of microflora of faeces and/or whole mid/hindgut of *L. vannamei*

The abundance and size distribution of 16S rDNA terminal restriction fragments (TRFs) obtained from faecal and whole mid/hindgut samples during the feed trial exhibited significant variation both between samples (pairs of animals) and diet groups (Figures 7 & 8). The samples collected from all three diet groups at week 4 exhibited a fragment distribution profile similar to that displayed by the baseline (Figures 7 & 8). There was no apparent divergence in peak distribution between the diet groups after 4 weeks of feed administration, as indicated by principal component analysis; no clustering of sample data was observed (Figure 9). All three diet groups displayed the highest abundances of fragments at 104, 107 and 306 base pairs, the control and chitin only diet groups also exhibited larger peaks at 667 base pairs compared to those of the baseline and *V. gazogenes* plus chitin samples.

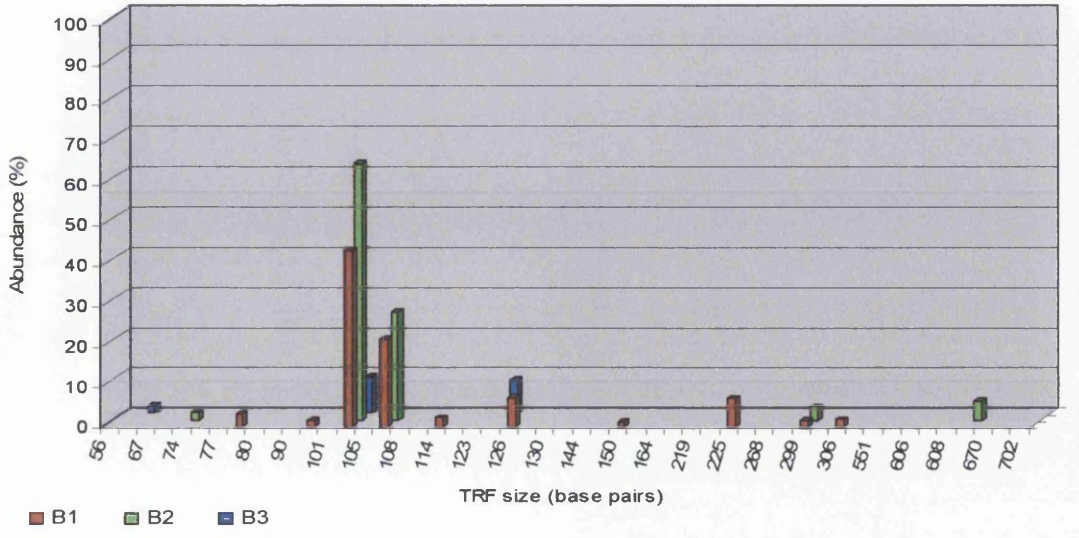


Figure 7. Size distribution profiles of T-RFLP fragments from baseline faecal samples (B1-B3) collected from the mid/hindgut of Pacific white shrimp *Litopenaeus vannamei*

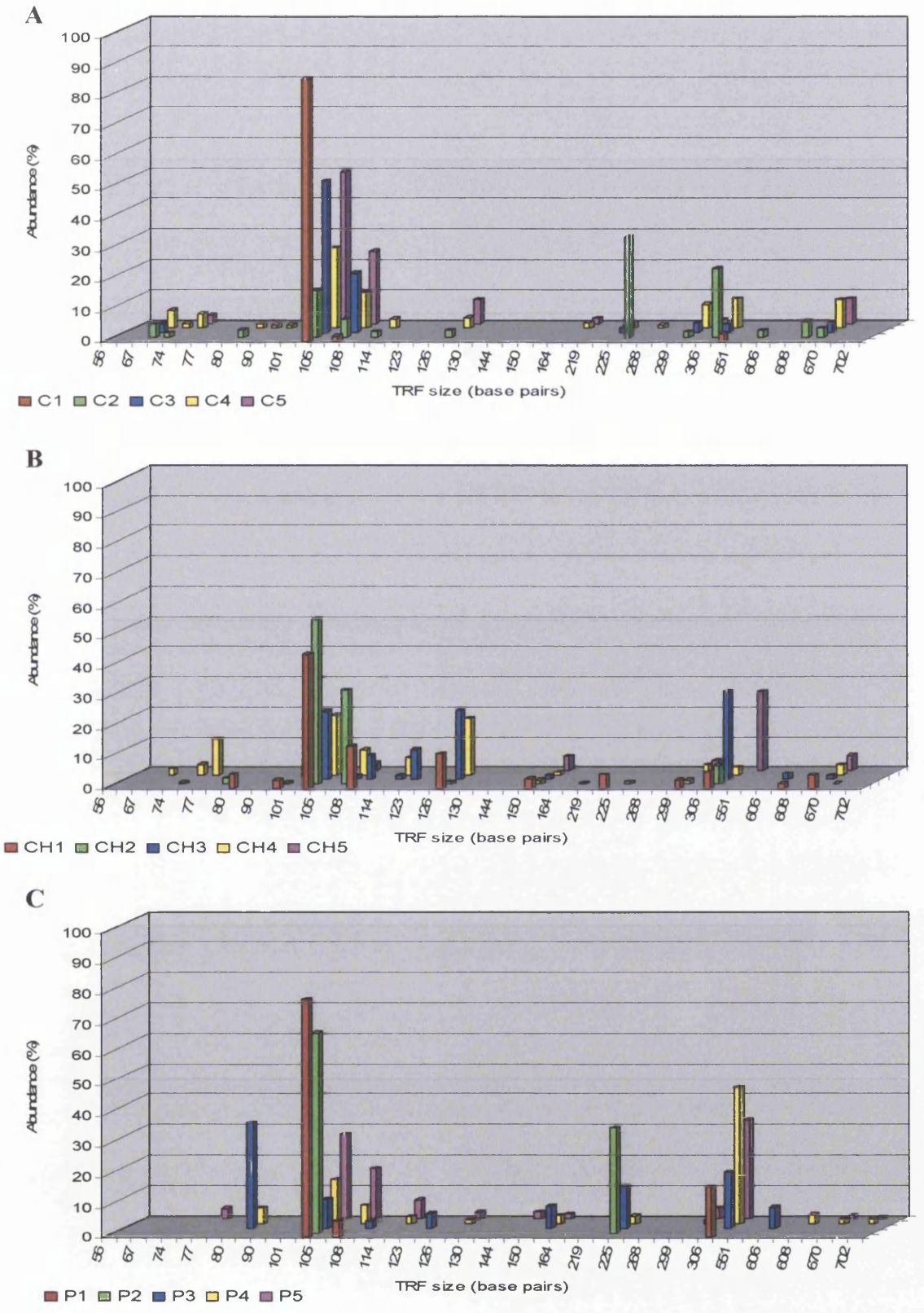


Figure 8. Size distribution profiles of T-RFLP fragments obtained from faecal samples collected from the mid/hindgut of Pacific white shrimp, *Litopenaeus vannamei*, after 4 weeks receiving; (**A**) a control diet (C1-C5); (**B**) a chitin supplemented diet (CH1-CH5); (**C**) a *Vibrio gazogenes* plus chitin supplemented diet (P1-P5).

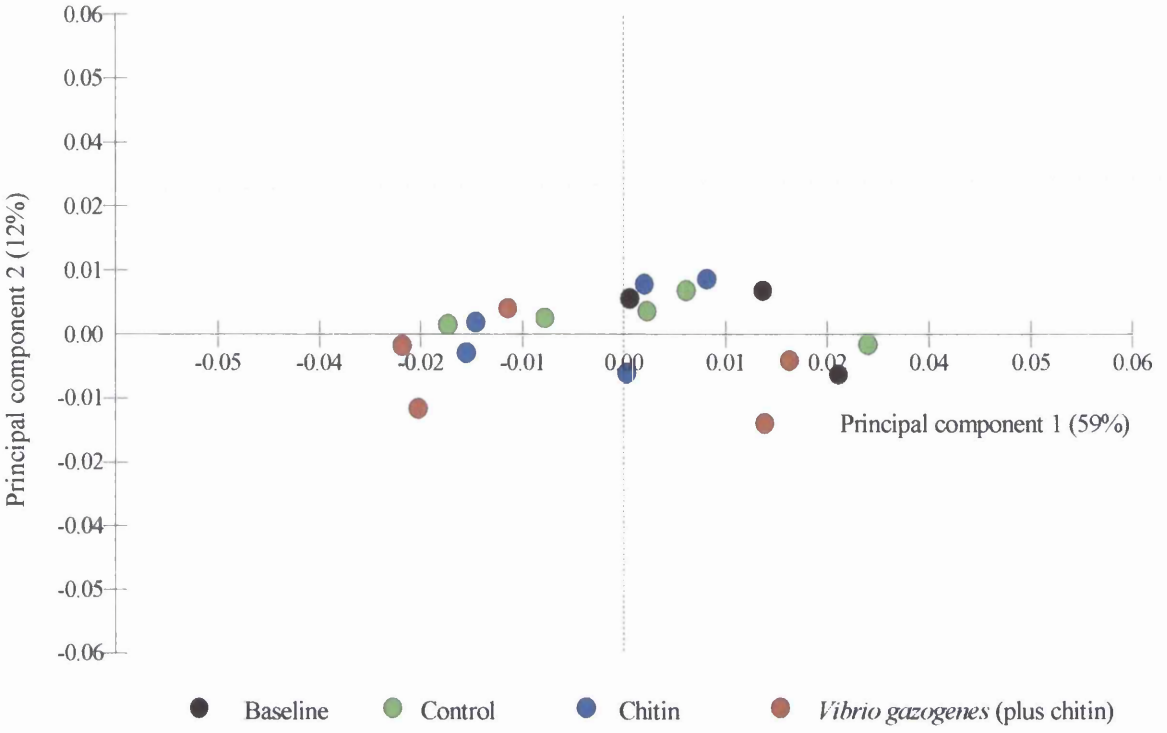


Figure 9. PCA ordination of bacterial T-RFLP peak height data for week 4 faecal samples from control, chitin supplemented and *V. gazogenes* plus chitin supplemented diet groups (baseline samples included).

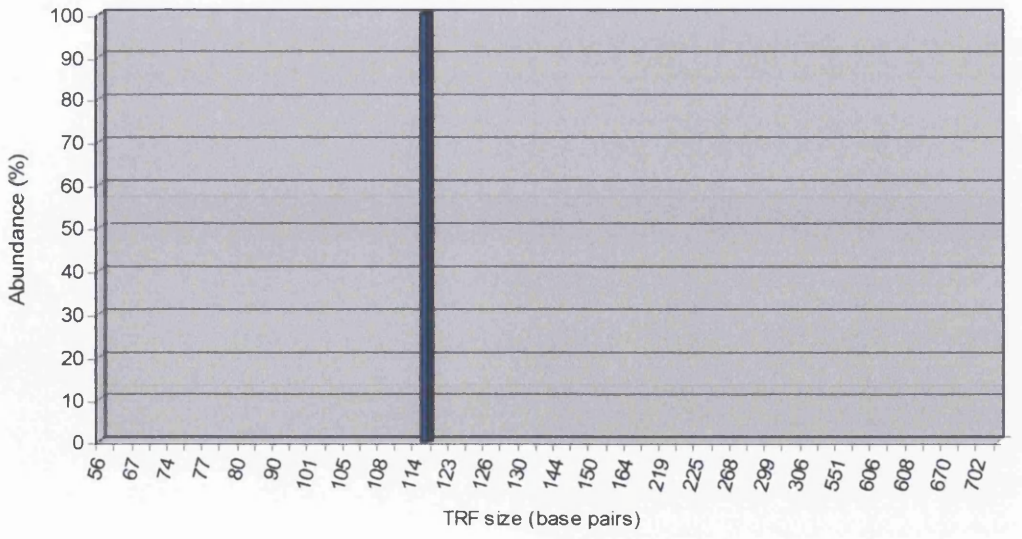


Figure 10. Size distribution profile of T-RFLP fragments obtained using restriction endonuclease *Hph*1 for potential probiotic *Vibrio gazogenes*.

Small abundance peaks for a TRF of 114 base pairs (the same fragment size displayed in the *V. gazogenes* TRF profile obtained using *Hph*1 enzyme digest; Figure 10) were observed in samples P1, P4 and P5 of the *V. gazogenes* supplemented diet group samples (Figure 11C). Such peaks were absent from the sample profiles of the chitin supplemented diet group (Figure 11B) but were observed in a baseline sample, B1, and in week 8 control group sample, C5 (Figures 7 and 11A). At week 8, all three diet groups exhibited the highest abundance of TRF at 126 and 306 base pairs; they also displayed smaller peaks at 607 base pairs, although not consistently over all samples (Figure 11). The control and chitin supplemented diet groups exhibited the greatest abundance (averaged at 65%) of fragments at 306 base pairs (Figure 11A & B). The group receiving *V. gazogenes* exhibited the greatest abundance of fragments (averaged at 57%) at 126 base pairs (Figure 11C). A separate PCA was performed on the week 8 T-RFLP peak height data as the samples collected during week 8 were of whole mid/hindgut as opposed to merely mid/hindgut contents as was analysed from week four. The baseline values were excluded from the week 8 sample analysis for the same reason. The peak height (% abundance) values and PCA analysis both indicated a difference between the mid/hindgut microflora of the *V. gazogenes* plus chitin diet group and those of the control and chitin supplemented diet groups, at week 8; indicated by the apparent separation and clustering of the probiotic fed group (Figure 12). In addition, the PCA illuminated no discernible (statistically significant) differences triggered by the variables represented by the first two principal components in the TRF distribution profiles of the control and chitin supplemented diet groups (Figure 12).

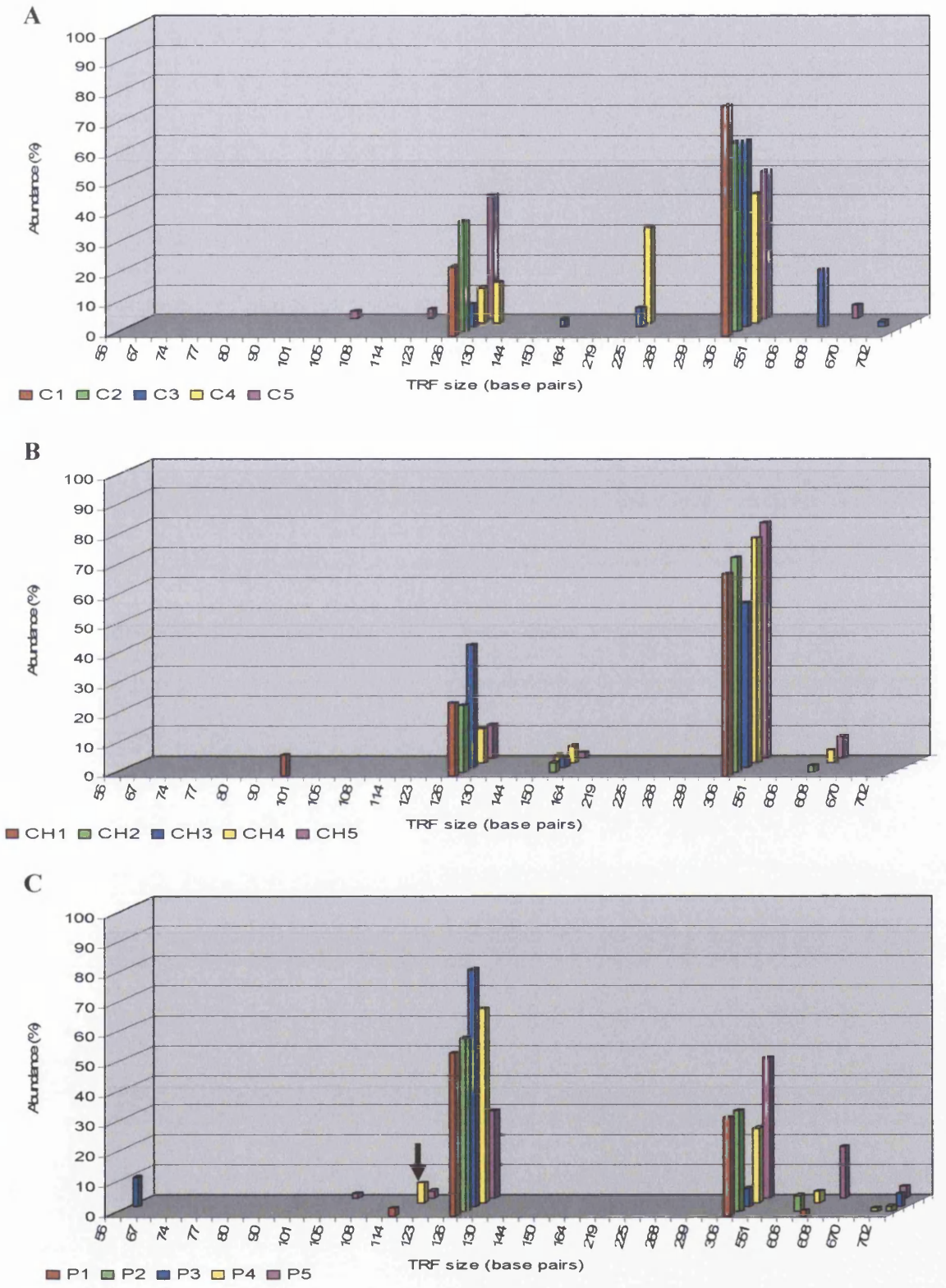


Figure 11. Size distribution profiles of T-RFLP fragments obtained from faecal samples collected from the mid/hindgut of Pacific white shrimp, *Litopenaeus vannamei*, after 8 weeks receiving; (**A**) a control diet (C1-C5); (**B**) a chitin supplemented diet (CH1-CH5); (**C**) a *Vibrio gazogenes* plus chitin supplemented diet (P1-P5). Arrow indicates 114 base pair fragment size corresponding to the *V. gazogenes* profile.

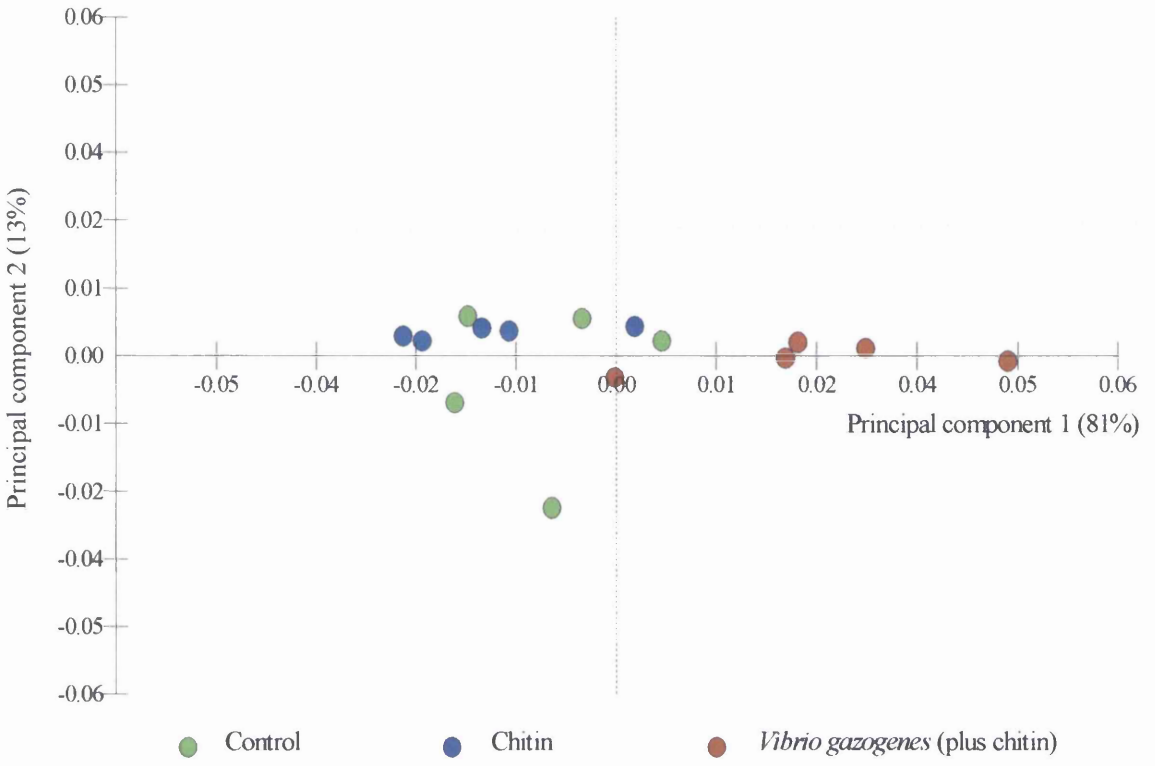


Figure 12. PCA ordination of bacterial T-RFLP peak height data for week 8 faecal samples from control, chitin supplemented and *V. gazogenes* plus chitin supplemented diet groups.

5.3.4 Trial 4: A 6-week multi-species, *Vibrio gazogenes* (NCIMB 2250) plus *Lactobacillus plantarum* feed trial in *Litopenaeus vannamei*

5.3.4.a Haemocyte counts

No statistically significant variation in total circulating haemocyte numbers was detected between the mono-strain (*V. gazogenes*) and the multi-species (*V. gazogenes* & *L. plantarum*) diet groups after 6 weeks of feed administration (Figure 13). Nor were any significant differences observed between the experimental diet groups' data and the baseline THC values.

Figures 13 through to 16 also illustrate that no statistically significant differences ($P>0.05$) existed between the two diet groups, or between the diet groups and the baseline data, with regard to the proportions of different circulating haemocyte types.

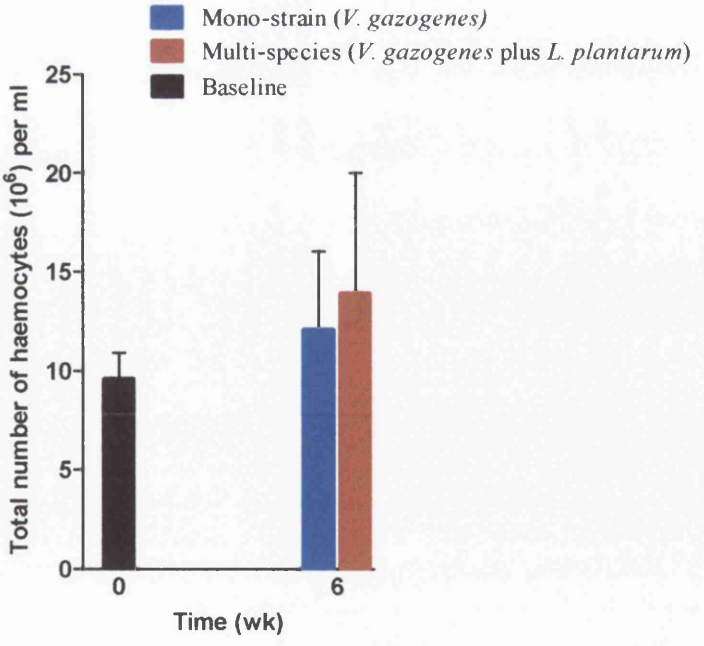


Figure 13. Total haemocyte numbers observed in haemolymph samples from baseline animals as well as those from the mono-strain (*Vibrio gazogenes* only) and multi-species (*V. gazogenes* plus *Lactobacillus plantarum*) diet groups after 6 weeks. Mean ± SEM, n=5.

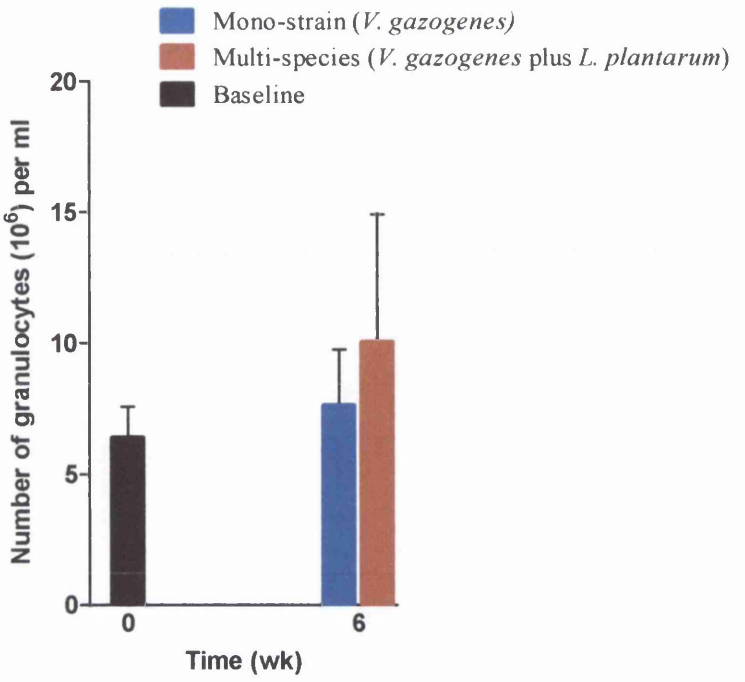


Figure 14. Number of granulocyte type haemocytes observed in haemolymph samples from baseline animals as well as those from the mono-strain (*Vibrio gazogenes* only) and multi-species (*V. gazogenes* plus *Lactobacillus plantarum*) diet groups after 6 weeks. Mean ± SEM, n=5.

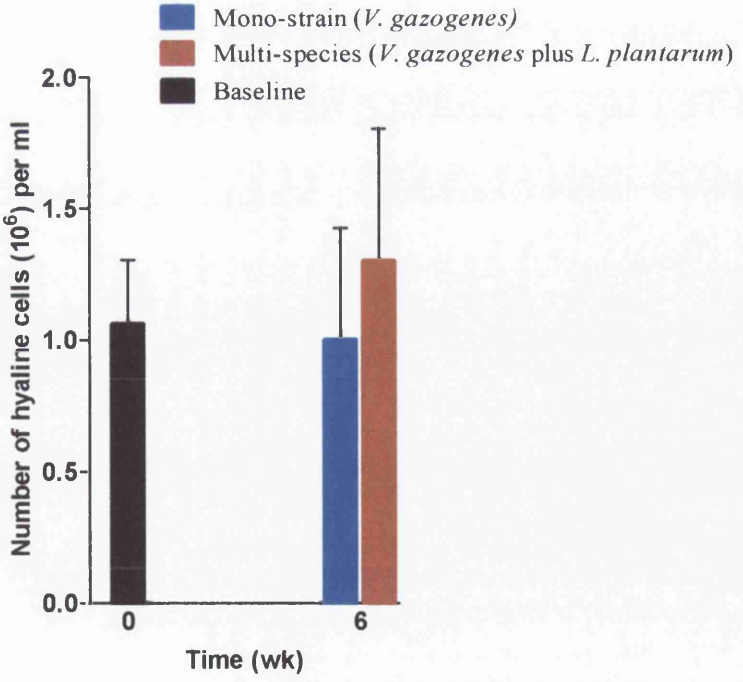


Figure 15. Number of hyaline type haemocytes observed in haemolymph samples from baseline animals as well as those from the mono-strain (*Vibrio gazogenes* only) and multi-species (*V. gazogenes* plus *Lactobacillus plantarum*) diet groups after 6 weeks. Mean ± SEM, n=5.

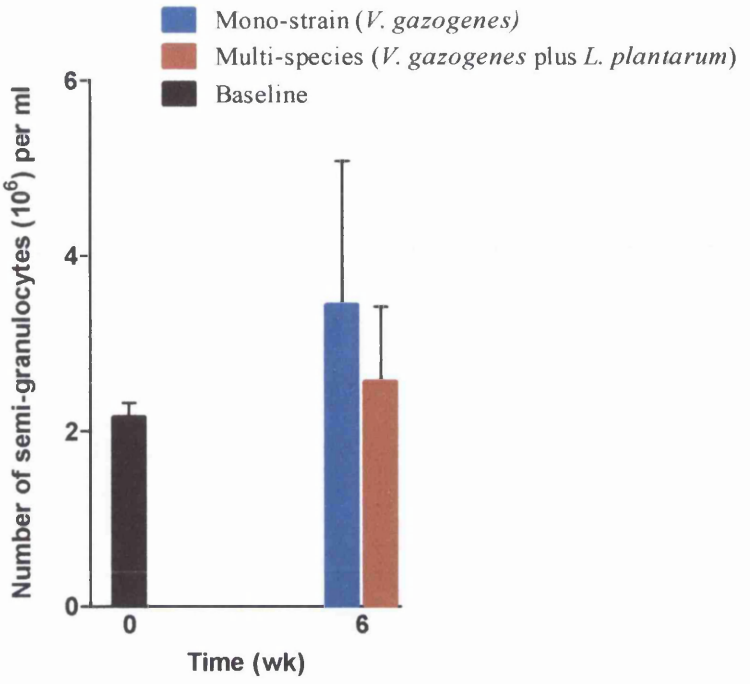


Figure 16. Number of semigranulocyte type haemocytes observed in haemolymph samples from baseline animals as well as those from the mono-strain (*Vibrio gazogenes* only) and multi-species (*V. gazogenes* plus *Lactobacillus plantarum*) diet groups after 6 weeks. Mean ± SEM, n=5.

5.3.4.b Mid/hindgut content microbiology

Statistically significant differences were observed in the total mid/hindgut content CFU data, however, no such differences, between any of the diet groups, were present amongst the *Vibrio*-like mid/hindgut CFU values (Table 4). Average total colony forming units present in the 6 week mid/hindgut content samples of the mono-strain diet group were significantly lower ($P<0.05$, Kruskal-Wallis test with Dunn's multiple comparisons post test) than those observed for the baseline and multi-species diet group samples. There was, however, no corresponding statistically significant decrease in the mean *Vibrio*-like mid/hindgut CFU values ($P>0.05$). Consequently, the proportion of the total colony forming units with *Vibrio*-like growth characteristic was significantly higher ($P<0.05$, Kruskal-Wallis test with Dunn's multiple comparisons post test) in the mono-strain diet group than in the baseline and multi-species diet groups (Table 4). No statistically significant variation in colony forming unit values was detected between baseline samples and those of the multi-species diet group.

Table 4. Total aerobic bacterial and aerobic *Vibrio*-like bacterial colony forming units present in mid/hindgut contents obtained from *Litopenaeus vannamei* receiving mono-strain (*Vibrio gazogenes*) or multi-species (*V. gazogenes* plus *Lactobacillus plantarum*) diets. (Mean ± SEM, n=5, * $P<0.05$ compared with baseline; * $P<0.05$ compared with Monostrain).

Diet type administered	Period of feed administration	Total bacterial content of hindgut (CFU g^{-1} wet weight)	*Vibrio* -like bacterial content of hindgut (CFU g^{-1} wet weight)	% of total culturable bacteria with *Vibrio* -like growth characteristics
Commercial†	Baseline (0 wk)	$3.41 \pm 0.75 \times 10^{8}$	$0.36 \pm 0.11 \times 10^{6}$	0.08 ± 0.02
Mono-strain (*V. gazogenes* only)	6 weeks	$0.53 \pm 0.07 \times 10^{8}$ *	$0.18 \pm 0.05 \times 10^{6}$	0.37 ± 0.10 *
Multi-species (*V. gazogenes* & *L. plantarum*)	6 weeks	$2.31 \pm 0.52 \times 10^{8}$ *	$0.52 \pm 0.29 \times 10^{6}$	0.15 ± 0.05 *

† Dragon Feeds Supreme™.

5.3.4.c Hepatosomatic Index

Both the mono-strain and multi-species diet groups exhibited higher hepatosomatic index values (at week 6) than those recorded during the baseline sampling. Despite this, these differences and those between the two diet groups were not statistically significant (Figure 17).

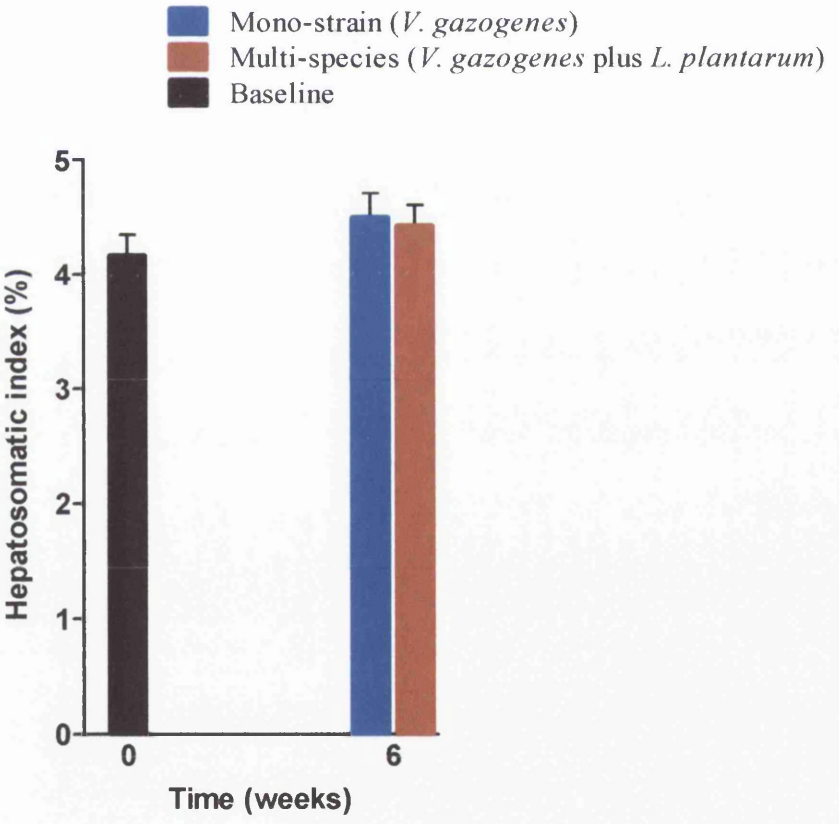


Figure 17. Hepatosomatic index of baseline animals as well as those of the mono-strain (*Vibrio gazogenes* only) and multi-species (*V. gazogenes* plus *Lactobacillus plantarum*) diet groups. Mean ± SEM, n=10.

5.3.4.d 16S rDNA T-RFLP analysis of whole mid/hindgut microflora of *L. vannamei*

The terminal restriction fragment abundance profiles for baseline, mono-strain (*V. gazogenes*) and multi-species (*V. gazogenes* plus *L. plantarum*) diet groups all exhibited peaks at fragments sizes of 114, 126, 306 and 607 base pairs (Figures 18A-C). The largest of these in the baseline and mono-strain samples were for TRFs of 126 and 306 base pairs. Three of the mono-strain samples displayed small peaks at 104/5 base pairs; these were not present in either the baseline or multi-species samples. The multi-species samples exhibited the greatest TRF abundance at 130 base pairs as well as a secondary peak at 275 base pairs (neither of which were detected in samples from the other groups) (Figure 18C). The absence of peaks for TRFs of 130 and 275 base pairs indicated that the mid/hindgut microfloral ecology of the multi-species diet group had been altered and was noticeably different to that of the baseline and mono-strain diet group. This is further reinforced by the results obtained for the principal component analysis, which showed a distinct clustering of the multi-species group samples away from those of the baseline and mono-strain group (Figure 20). The TRF peaks at 130 and 275 base pairs correspond to the T-RFLP peak abundance profile for *L. plantarum* (Figure 19).

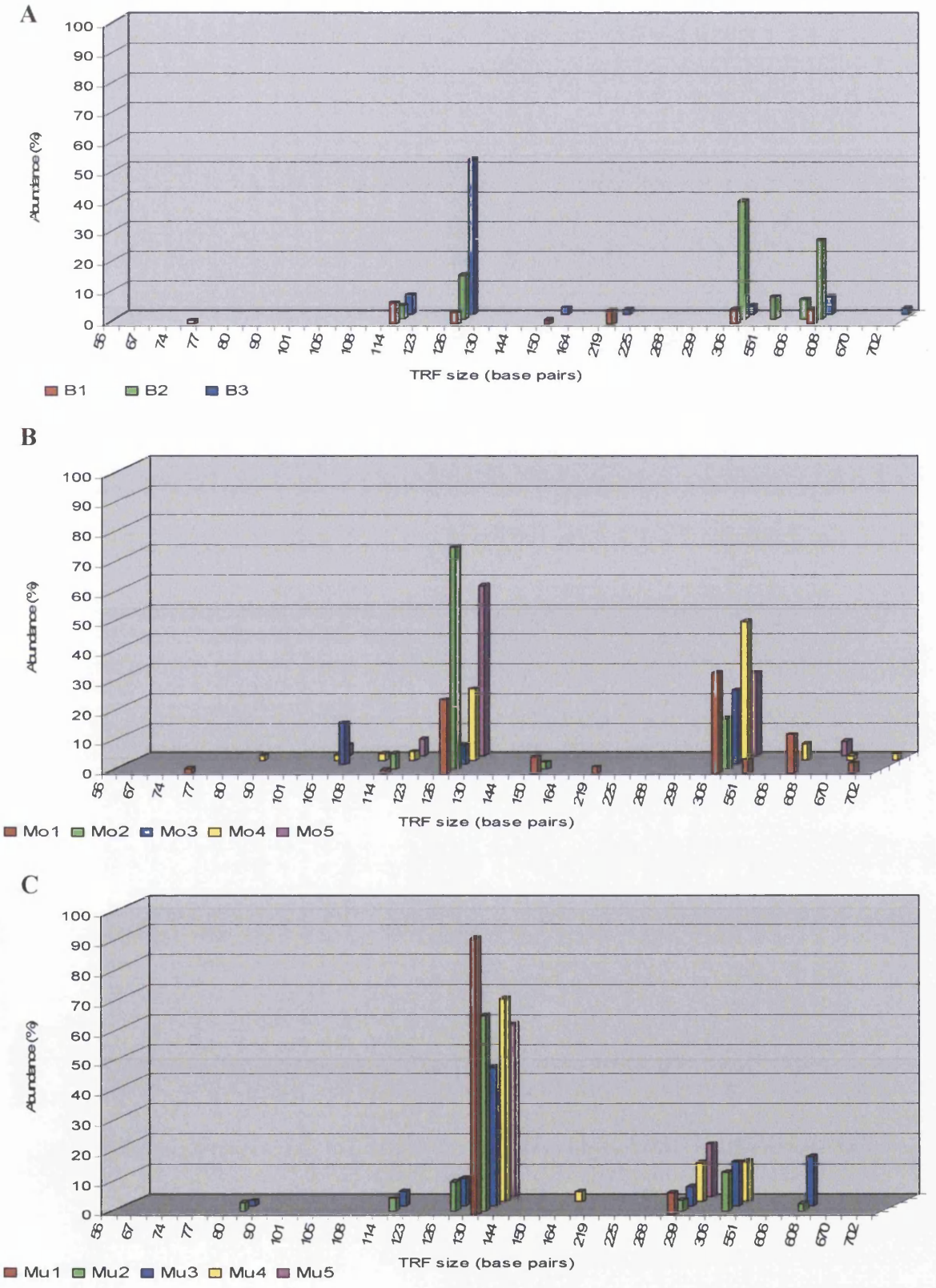


Figure 18. Size distribution profiles of T-RFLP fragments obtained from faecal samples collected from the mid/hindgut of Pacific white shrimp, *Litopenaeus vannamei*; (**A**) baseline (B1-B3); (**B**) after 6 weeks receiving a Mono-strain (*V. gazogenes*) diet (Mo1-Mo5); (**C**) after 6 weeks receiving a Multi-species (*V. gazogenes* & *L. plantarum*) diet (Mu1-Mu5).

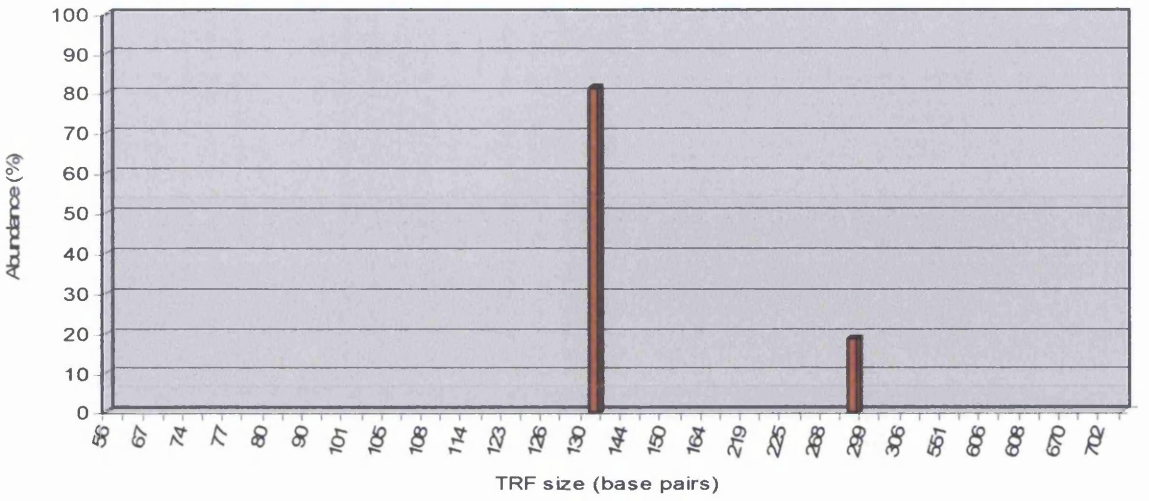


Figure 19. Size distribution profile of T-RFLP fragments obtained using restriction endonuclease *Hph*1 for lyophilised *Lactobacillus plantarum*.

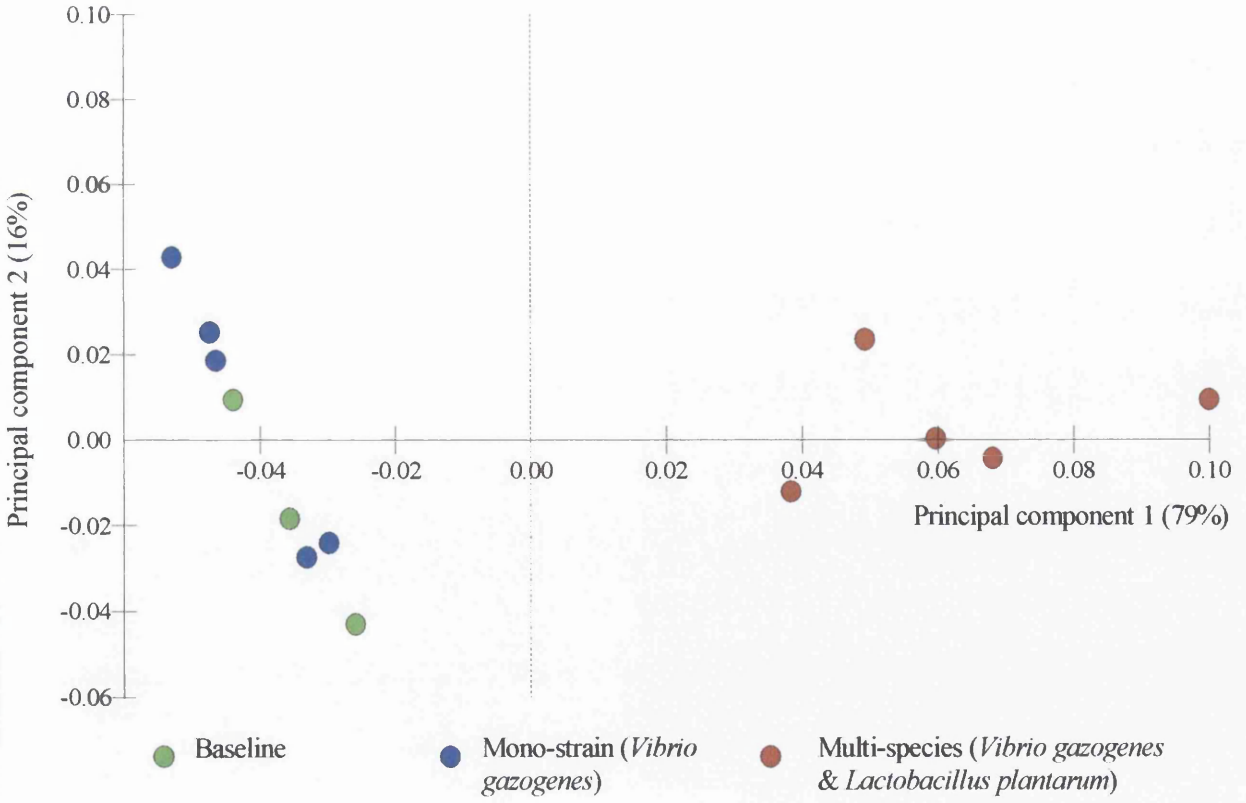


Figure 20. PCA ordination of bacterial T-RFLP peak height data for week 6 whole mid/hindgut samples from mono-strain (*V. gazogenes* supplemented) and multi-species (*V. gazogenes* plus *L. plantarum* supplemented) diet groups (baseline samples included)

5.4 Discussion

The assessment of the *in vivo* efficacy and mode/modes of action of potentially probiotic micro-organisms is often extremely problematic (Verschuere et al, 2000; Irianto & Austin, 2002). As described in Chapter 1, potential probiotics may benefit the host organisms via one, or more, methods. Consequently, in this chapter the sampling protocols were optimised to collected data on a wide range of parameters which could theoretically be used to elucidate as many potential modes of action as possible. It was impractical, however, to include the examination of growth and feed utilisation parameters within a feed trial necessitating the sacrifice of animals for tissue samples, for the simple reason that the former requires that the animals remain alive for the duration of the trial. Thus, several feed trials were required to assess the effects on *L. vannamei* upon the administration of *V. gazogenes* and *L. plantarum.*

Trial 1

The first trial undertaken was preliminary with regards to methodology and scope (Sections 5.2.1.1 & 5.3.1). Its primary function was to ascertain whether *V. gazogenes* was safe for oral administration to *L. vannamei.* In addition, at the cessation of the trial the animals were then utilised in development of sampling methods used in later trials. The success of the trial in its primary aim is undetermined, as no *V. gazogenes* colonies were recovered from samples of the top-coated diet. Consequently, there is no evidence to indicate the animals actually ingested live *V. gazogenes.* Initial screening work on *V. gazogenes* (Chapter 3, Section 3.3.3), however, indicated that *V. gazogenes* retained a significant proportion of its *Vibrio* antagonistic potential when dead. Therefore, it was concluded that viable *V. gazogenes* were not required in order

to potentially elicit an effect in *L. vannamei.* Furthermore, the definition of a probiotic has been extensively expanded from that outlined by Fuller (1987) and now encompasses the products, derivatives and components of microorganisms as well as the organisms themselves (Kesarcodi-Watson et al, 2008). Accordingly, the decision was taken to proceed with feed trials using a top-coated *V. gazogenes* diet despite being unable to recover viable CFU from said feed. It was also decided to continue the administration of powdered chitin, as a second variable, alongside *V. gazogenes* in future feed trials. Although chitin was originally included in the diet in an attempt to stabilise and maintain the viability of the potential probiotic, it is suggested to have the capability to purge potential bacterial (*Vibrio* spp.) pathogens as it transits the crustacean G.I tract (Powell & Rowley, 2007). The effects of chitin administration on the microbial diversity of the crustacean mid/hindgut had never been assessed via molecular means, such as T-RFLP.

Trial 2

Trial 2 (Section 5.3.2) investigated whether the oral administration of *L. plantarum* (via top-coated feed) conveyed any benefit to post-larval *L. vannamei* with regards to growth and/or feed utilisation. None of the variations observed in the recorded parameters between the control and *L. plantarum* diet groups over the 28 day feeding period were statistically significant. However, it is likely that any benefits wrought by *L. plantarum* would be cumulative and gradual and therefore may only become apparent over longer-term administration. In conclusion, the length of Trial 2 may have been insufficient for the detection of any growth benefit to *L. vannamei.* The trial period of 28 days was selected after the review of a previous study (Balcázar et al, 2007) which indicated that probiotic administration significantly enhanced growth

and feed conversion rates in *L. vannamei.* Supplementation with *L. plantarum* had also been shown to increase resistance to the pathogen *Vibrio alginolyticus* in shrimp, after only 7 days administration (Chiu et al, 2007). In conclusion, however, the results obtained for *L. plantarum* in this trial do not mirror those observed for the probiotics utilised by Balcázar et al (2007) with regard to enhancement of short-term growth performance in *L. vannamei.*

Trial 3

The aim of the third trial (Trial 3; Section 5.3.3) was to determine whether the administration of a feed top-coated with powdered chitin, impregnated or not, with *V. gazogenes* produced any change in circulating haemocyte populations, the microbiotal ecology of the mid/hindgut or the health/nutritional status (hepatosomatic index) of *L. vannamei.* As in the first feed trial, live *V. gazogenes* was unrecoverable from the finished feed. A statistically significant variation in the total circulating haemocyte population was observed occurring in the control diet groups at the four week sample point. At week 4 the control group displayed significantly higher numbers of circulating granulocyte-type haemocytes and hyaline cells than were observed in the baseline animals (t_0). These haemocyte cell populations were not, however, significantly elevated in the control group data gathered in week 8. The week 4 count data for the chitin and *V. gazogenes* diet groups displayed no significant variation when compared to the baseline values with one exception; the week 4, chitin group granulocyte-type cell count was significantly elevated compared to the baseline. The week 8 chitin diet group haemocyte counts were higher than those of the baseline and corresponding control, but were not significantly so, indicating that the administration of chitin had no discernible effect on circulating haemocyte populations. Overall,

although there were some significant changes in haemocyte numbers, there is no clear evidence of any systematic effect of either chitin or *V. gazogenes*. This is unlike other reports, where the oral administration of *L. plantarum* was observed to significantly lower THC in *L. vannamei* (Chui et al, 2007) and in Li et al (2008) where injection of shrimp with *V. alginolyticus* produced a similar effect.

The control diet group also displayed a statistically significant lower average HSI value at week 4 when compared to the baseline samples and the chitin and *V. gazogenes* (plus chitin) diet groups. This difference was not observed at week 8 however. Other studies have shown that probiotic supplementation affects HSI values (e.g. Castex et al, 2008). Castex et al (2008) showed the administration of *P. acidilactici* over 5 weeks increased the HSI of juvenile *L. vannamei* by 10%.

Examination of the bacterial population of the mid/hindgut was undertaken via two methods. Firstly, basic microbiological techniques were used to determine the number of viable total and *Vibrio*-like microorganisims present in the faeces. Of greatest significance were the variations in the proportion of CFU with *Vibrio*-like growth characteristics between the diet groups. By week 8 both experimental diet groups (i.e. animals receiving chitin or chitin plus *V. gazogenes*) displayed statistically significant decreases in the proportion of *Vibrio*-like microbes present in samples of mid/hindgut contents, compared to the baseline samples. In addition, in week 4 the *V. gazogenes* diet group also exhibited a significantly lower proportion of *Vibrio*-like CFU than the corresponding control samples. Therefore, it could be concluded that the chitin ingested by the animals in the two experimental diet groups performed a 'purge' of *Vibrio*-like microorganisms from the G.I. tract, confirming the findings of Powell & Rowley (2007) who performed a similar experiment in the shore crab, *Carcinus maenas*. A further observation of note with regard to the mid/hindgut content

microbiology data was an apparent decrease in the variability of the proportion of *Vibrio*-like microorganisms within those diet groups receiving chitin when compared to the baseline and corresponding control groups. This may indicate that the administration of chitin may also reduce the variability of the gut microflora of shrimp receiving such a diet. This appears at least partially supported by the TRF profiles for week 8 (Section 5.3.3.d) where the majority of DNA fragments were of two sizes (126 & 306 base pairs). However, care must be taken in drawing such a conclusion as the sample collection methodology used in the T-RFLP analysis was altered between weeks 4 and 8.

A more detailed picture of mid/hindgut bacterial ecology was produced via T-RFLP analysis. The peak size distribution profiles of 16S terminal restriction fragments gives an indication of the microbial diversity within a sample, simply, the greater the number of fragments (peaks) the greater the microbial diversity (Marsh, 1999; Kitts, 2001). The TRF distribution profiles obtained in Trial 3 displayed little variation in the number of fragment sizes (peaks) present at each sample period; the greatest variation occurred with regard to the relative abundance (i.e. size) of these peaks. Microbial diversity appeared to be highest in the week 4 samples compared to those from week 8. However, this is likely the result of the change in sampling protocol, i.e. the analysis of whole mid/hindgut (including contents) in week 8 as opposed to solely extruded gut contents in week 4. Examination of the peak size distribution profiles of T-RFLP fragments obtained from mid/hindgut samples coupled with principal component analysis (PCA) indicated variation in the mid/hindgut bacterial ecology of the diet groups. PCA conducted on the week 4 data indicated no evidence of clustering of individual diet groups and no significant variation from the baseline TRF profile. However, the PCA conducted on the 8 week mid/hindgut samples, showed the

points representing the *V. gazogenes* diet group clustered separately from those of the control and chitin groups, on the first principal component axis. This indicated that the mid/hindgut microflora population ecology of animals in the *V. gazogenes* group was significantly different compared to the control and chitin diet groups after 8 weeks receiving the *V. gazogenes* supplemented diet. In addition, three of the five TRF profiles representing the *V. gazogenes* diet group samples exhibited a minor peak at 114 base pairs; this peak corresponded to the 16S fragment size for the *V. gazogenes* TRF profile (for the *Hph*1 enzyme digest). Superficially, this would seem to indicate the presence of a limited amount of *V. gazogenes* in the samples. However, other closely related bacteria share this 16S fragment size, for example *V. vulnificus* and *Listonella anguillarum* (formerly *Vibrio anguillarum*). Both of these bacteria have also been observed in gut/gill microbiota of penaeid shrimp (Lightner, 1983; Vaseeharan & Ramasamy, 2004; Ruangpan & Kitao, 2006; Longyant et al, 2008). The presence of 114 base pair fragments in very similar abundances in the profiles of baseline and week 8 control group samples, 'B1' and 'C5', coupled with the inability to recover live *V. gazogenes* from the feed, would suggest that the peaks observed in the *V. gazogenes* diet group profiles may also represent one or more of these related species. The week 8 TRF profiles of the three diet groups exhibited a very similar pattern with regard to peak distribution, however, the *V. gazogenes* diet group displayed a noticeable difference in the weighting between these peaks, with regard to fragment abundance. It was this variation that was detected by the week 8 PCA and resulted in the separate clustering of the *V. gazogenes* diet group. Subsequently, given the absence of live *V. gazogenes* in the feed and apparent absence or limited presence of the microorganism's DNA profile from the T-RFLP analysis, it is impossible to determine what factor/s resulted in this variation. This is a weakness of the approach

taken, using T-RFLP rather than, for example, denaturing gradient gel electrophoresis (DGGE). With DGGE there is the ability to obtain sequence data (and therefore taxonomic information) from bands (DNA fragments) cut from the gel. The week 8 PCA did not indicate any significant variation in mid/hindgut bacterial diversity between animals receiving the control and chitin supplemented diets. Surprisingly, the statistically significant drop in the proportion of *Vibrio*-like microorganisms present the in mid/hindgut contents, detected using standard plate counting, for those animals receiving supplemental chitin, did not result in a separate clustering of the chitin diet group data during PCA. This may be due to the variations in the sampling methodology. The samples utilised for the week 8 T-RFLP were of whole mid/hindgut, where as those used for the faecal microbiology work were of extruded mid/hindgut contents. Consequently, it could be argued that given the chitin binding tendencies of *Vibrio* spp. (Powell & Rowley, 2007) such organisms in the gut contents would exhibit increased binding to the fragments of supplemental chitin present, thereby reducing the proportion of *Vibrio*-like organisms available to colonise the media. This would of course also occur in the whole mid/hindgut samples; however, the overall impact would be lessened by the presence of *Vibrio* spp. adhered to the chitinous hindgut wall.

In conclusion, T-RFLP may not be an optimum method for assessing the variations in the G.I microbial populations of animals receiving microbial supplements. The technique has both strengths and weaknesses with regard to the microbial community analysis attempted in Trials 3 & 4. T-RFLP can be extremely effective when used to compare the microbial community composition of different samples without the need to culture (Thies, 2006). This was particularly useful in this project given the number of samples and the limited time frame. Therefore, a key weakness of the technique is

its inability to often distinguish between related taxa (Thies, 2006). Consequently, the addition of large quantities of microbial DNA (i.e. in the form of a probiotic bacterium) will of course alter the microbial composition (TRF profile) and such an alteration would be illuminated by the subsequent PCA. The grouping of related taxa and the displaying of the terminal restriction fragments detected as relative abundances (rather than discrete empirical values) ensures that the removal of the peak/s appearing to corresponding to a specific microorganism (i.e. a probiotic) is impossible without the potential removal/alteration of the profiles of other closely related naturally occurring species. The removal of such related taxa would also likely result in significant differences between sample profiles (compared to baseline or controls) being (falsely) detected, thereby negating any potential advantage in attempting to remove peaks appearing to corresponding to the probiotic/s. To summarise, T-RFLP is a powerful technique in determining differences in microbial community composition between samples where the grouping variable is non-microbial in nature, e.g. a potential immunostimulant of non-microbial origin such as chitin, or an abiotic factor such as temperature, media composition, pH, etc. The efficacy of T-RFLP in assessing the effects of bacterial supplementation on G.I. bacterial community composition is, however, more questionable.

Trial 4

The final feed trial (Trial 4) was conceived as a preliminary study examining the potential of using mixtures of beneficial microorganisms (multi-strain/species supplements) as opposed to a single or mono-strain supplement. Previous research has given strong indication that the probiotic potential (and thus benefits) of multi-strain/species supplements may be far greater than that of bacterial strains

administered singularly (Timmerman et al, 2004). Given that this trial was a direct comparison and that the effects of a *V. gazogenes* plus chitin diet had already been assessed alongside a chitin supplemented and control diet, only mono-strain (*V. gazogenes*) and multi-species (*V. gazogenes* plus *L. plantarum*) diet groups were assessed. This strategy was also selected due to tank space in the CSAR Facility being highly restricted at the time the trial was undertaken. No statistically significant variation in circulating haemocyte numbers/populations or in nutritional status (HSI) was observed between the diet groups or between the diet groups and the baseline samples. This was unexpected as Castex et al (2008) showed a significant increase in the HSI of *L. stylirostris* of the same weight administered the lactic acid bacteria, *P. acidilactici*, after only 5 weeks of feeding. However, the trial described in Castex et al (2008) was conducted in a commercial pond setting rather than in a closely monitored re-circulation system. It is therefore likely that the animals assessed in the Castex and co-authors trial did not, at the commencement of feeding, possess a health or nutritional status equal to that of animals raised in the CSAR facility. The current THC data also appear to contradict that of Chui et al (2007) who indicated that *L. plantarum* administration in similar concentrations resulted in a significant decrease in total circulating haemocyte numbers in adult *L. vannamei.*

The mid/hindgut content microbiology data collected showed a large amount of variation in the total bacteria counts of the mono-strain diet group. However, the numbers of Vibrio-like CFU present in the mono-strain diet group samples were not significantly different to those of the baseline and multi-species groups. Given that a *V. gazogenes* supplemented diet administered in the previous trial initiated no such decline in total mid/hindgut CFU, this variation is likely due to variables introduced during the collection and processing of the samples. In addition the data collected did

not support the findings of Castex et al (2008) which showed a significant drop in both total and *Vibrio* CFU after only three weeks of *P. acidilactici* supplementation. The administration of *L. plantarum* which exhibited very similar *in vitro* anti-*Vibrio* activity to *P. acidilactici* in Chapter 3 resulted in no such reduction. The data are, however, partially supported by Vieira et al (2008) who found no significant changes in total digestive tract bacterial counts in *L. vannamei* after administration of *L. plantarum*. Vieira et al (2008) did, however, find that the numbers of culturable *Vibrio* spp. present in the G.I. tract decreased significantly after receiving *L. plantarum*.

T-RFLP and subsequent PCA indicated that the composition of the mid/hindgut bacterial community of animals receiving the multi-species diet, was different from the baseline and mono-strain diet communities. This was indicated by a definitive clustering of the multi-species group separate from the baseline and mono-strain groups on the first principal component axis. The PCA of the mono-strain group appears to conflict with the data obtained in Trail 3. In this trial the mono-strain and baseline profiles are not clustered separately as in Trial 3. This deviation could be a result of numerous factors including; the different production techniques used and/or subtle variations in diet composition, which may have affected the microbial content of the feed. The absence of *V. gazogenes* from the mid/hindgut samples was indicated by the small and variably distributed peaks (at 114 base pairs) in the mono-strain and multi-species TRF profiles. The presence in the baseline profiles of peaks of the same size at 114 base pairs, gives further indication that *V. gazogenes* DNA was not represented in the diet group samples. Peaks for TRF's of 130 and 275 base pairs were restricted to the sample profiles of the multi-species diet group and corresponded to those observed in the TRF profile for pure culture *L. plantarum*. These peaks were

absent from the mono-strain and baseline profiles and accounted for a significant proportion of the 16S TRF. The PCA conducted on the TRF profiles of the diet groups and baseline samples indicated that there was no statistically significant variation in the mid/hindgut bacterial community compositions of the baseline and mono-strain diet groups. It did, however, show the multi-species diet group samples were clustered away from those of the baseline and mono-strain groups on the first principal component axis. This indicated that the composition of the mid/hindgut bacterial communities present in samples from the multi-species diet group were significantly different to those of the baseline and mono-strain samples. As outlined previous for Trial 3, however, a weakness of the T-RFLP technique and its analysis, is the inability to differentiate between profile differences caused by the presence of the probiotic supplement (in this case *L. plantarum*) or the effect the presence of said supplement has on bacterial community composition.

Conclusions

The main findings of this chapter are as follows:

1. The oral administration of the probiotic, *L. plantarum*, over a short (28 day) period, had no discernable effect on growth rate, feed conversion rate or survival in post-larval Pacific white shrimp, *L. vannamei.* In addition, the administration of *L. plantarum* in conjunction with *V. gazogenes* produced no significant change in the health/nutritional status of juvenile shrimp.

2. *L. vannamei* receiving the potential probiotic, *V. gazogenes*, showed no indications of enhancement of health/nutritional status, although there were

some indications of the modulation of the microbial community of the animals mid and hindguts.

3. The administration of chitin reduced the levels of *Vibrio*-like organisms in mid/hindgut contents of *L. vannamei.*

4. The use of T-RFLP in determining changes in the diversity of gut bacterial communities following the administration of probiotics was the primary weakness in this chapter. The inability to discern between changes wrought by the activity of the probiotics, and those brought about by their simple presence, was the major drawback to this technique. In hindsight the use of temperature gradient gel electrophoresis (TGGE) or denaturing gradient gel electrophoresis (DGGE) would have been more applicable and would have allowed for the possible sequencing/identification of the genera present.

Chapter 6

General discussion and future directions

General discussion

As described in Chapter 1, the primary problem facing intensive, penaeid aquaculture is the loss of stock (and subsequent profit) through disease (Skjermo & Vadstein, 1999). The main causative agents of disease outbreaks in juvenile and adult *L. vannamei* populations are viral, predominantly white spot syndrome virus (WSSV) and Taura virus syndrome (TVS) (Flegel, 1996; Phuoc et al, 2009; Lotz et al, 2005). Little can be done to curtail such viral outbreaks once established; however, the frequency of such outbreaks may be reduced via improvements in farm management and animal husbandry. Such improvements may include enhanced water quality, better feed utilisation and a reduction in physiological stress due to variations in water temperature, salinity, O_2 content, etc (Kautsky et al, 2000). Larval and post-larval shrimp, however, have increased susceptibity to bacterial pathogens compared to animals at later lifecycle stages (Hameed, 1993). A significant contributing factor in this increased susceptibility is likely the lack of the stable, healthy, established gut microbiota displayed by older animals (Timmermans, 1987; Skjermo & Vadstein, 1999; Hong et al, 2005). The usefulness of probiotic bacterial strains in increasing the health status of aquaculture species has been exhibited in finfish (Ringø & Gatesoupe, 1998; Irianto & Austin, 2002; Balcázar et al, 2006) and to a lesser extent in crustaceans (Rengpipat et al, 2003; Balcázar et al, 2006; Chiu et al, 2007). Although the primary efficacy of probiotics in a host appears to lie in countering bacterial pathogens (i.e. via competitive exclusion; Verschuere et al, 2000; Hong et al, 2005; Farzanfar, 2006; Van Hai et al, 2009), they may also have a role in lessening the frequency of viral outbreaks via improvement of the culture environment (i.e. bioremediation; Ninawe & Selvin, 2009) and in improving feed conversion rates (i.e.

via increased digestive efficiency; Castex et al, 2008; Gómez & Shen, 2008). Probiotics and immunostimulants are two of the most promising avenues for disease prevention currently available in penaeid aquaculture (Ninawe & Selvin, 2009). The problems associated with antimicrobials, primarily antibiotics, in shrimp aquaculture (outlined in Chapter 1) means that they can no longer be relied upon as a preventative measure with regard to bacterial disease outbreaks (Moriarty, 1999; Farzanfar, 2006). They remain, however, a viable method for the treatment of outbreaks once the pathogen has been identified. Therefore, the most practicable approach to disease management in an intensive, commercial, penaeid aquaculture facility appears to be a multifaceted one. Such an approach would encompass improved farm management techniques (i.e. better training and education of staff in the causes and means of disease prevention in shrimp, polyculture with detritivorous fish, use of pond liners, etc), in addition to the application of probiotics and immunostimulants (both in culture water and feed), bioremediation and the judicious use of effective antimicrobials (to treat bacterial disease outbreaks as and when they occur).

The data gathered during this project has displayed the (*in vitro*) ability of several species of bacteria to inhibit/interfere with the growth of potential penaeid, bacterial pathogens (Chapter 3). It has also indicated that a likely source of potential shrimp probiotics resides within the non-pathogenic *Vibrio* spp. and in those microbes previously utilised as terrestrial probiotics (e.g. *Bacillus* spp, *Lactobacillus* spp, *Pediococcus* spp, etc; Hong et al, 2005; Farzanfar, 2006; Chui et al, 2007; Castex et al, 2008). The inability during the project to derive a suitable, potential probiont from the pre-existing G.I. microflora of adult *L. vannamei* may be the result of the source of the animals screened rather than a general lack of such bacteria in shrimp microflora. The animals in question were hatched and raised in a closed recirculation

system; such systems contain depurated water with no contaminants or toxins and are continuously disinfected to eliminate potential pathogens (http://www.umbi.umd.edu/ 2009). Consequently, such a system will have a significantly less diverse microfloral community compared to that of *L. vannamei*'s natural habitat or an open pond (farm) environment. The G.I. microflora of an aquatic invertebrate appears greatly influenced by the microorganisms present in their feed and environment (Harris, 1993; Moss et al, 2000). Accordingly, the microfloral diversity of animals raised in such a highly regulated system would be considerably limited; thereby reducing the likelihood of isolating potential probionts. In hindsight, a more successful strategy may have been to screen the G.I. microflora of pond farmed/wild captured shrimp rather than those raised in such a system. However, the temporal and financial constraints of the project and the location of such farms (i.e. in the America's and S.E. Asia) made this impractical. This aspect also made the *in vivo* assessment of the efficacy of potential probionts problematic. The animals used in the trials described in Chapter 5 were raised and housed in the CSAR Facility (recirculation) systems; consequently, they were well fed and in excellent health at the commencement of *in vivo* testing. Any beneficial affects the potential probiotics/chitin may have had on the nutritional status (hepatosomatic index), immune parameters (THC & DHC) and indirectly via modulation of gut microbiota, would likely have been substantially mitigated by the animals initial excellent condition. However, there is little likelihood of closed/recirculation systems being widely adopted in commercial penaeid aquaculture (Dierberg & Kiattisimkul, 1996). The expense of such systems (both in terms of setup capital and running costs) coupled with the relatively low market return on farmed shrimp (compared to other aquatic animals), would be prohibitive. Consequently, closed/recirculation systems are largely restricted to research facilities,

or the smaller scale production of high value ornamental shrimp species (Calado et al, 2003). Any future *in vivo* research focused on the efficacy of probiotics/immunostimulants should be performed in a farmed pond setting (or a system setup to mimic the conditions found in such an environment). This is a weakness of the *in vivo* work conducted in this project, when compared to similar research in the current literature (i.e. Castex et al, 2008). An additional advantage in conducting such trials in a pond setting versus a recirculation system would be the increased space and abundance of experimental animals available as one of the primary constraints experienced during this project was a lack of available tank space within the CSAR facility. As well as the wider screening of microflora of pond farmed shrimp, further examination of non-pathogenic *Vibrio* spp. would also be merited given the anti-*Vibrio* activity exhibited by *V. alginolyticus* and *V. gazogenes* described in Chapter 3. A more detailed assessment of the probiotic activity of non-virulent strains of *V. alginolyticus* could also prove fruitful, given the high level of anti-*Vibrio* activity displayed by the strain assessed in this project and previous research indicating the species potential as a shrimp probiotic (Austin et al, 1995; Gomez-Gil et al, 2002). Any additional *in vitro* screening could also encompass members of the Bacillaceae for potential aquatic probionts (Hong et al, 2005). Future trial work would likely be rendered more robust by also having available the ability to challenge animals receiving probiotic/immunostimulant supplementation (Castex et al, 2008).

The nature of the aquatic invertebrate G.I. tract makes the administration of bacterial supplements via feed less effective than in terrestrial animals and finfish (Gatesoupe, 1999; Skjermo & Vadstein, 1999). The apparent transient nature of the G.I. microflora of many species of crustacean would indicate that the administration of beneficial

bacteria via culture water would likely be a more efficient (and cost effective) means of supplying beneficial microorganisms (Harris, 1993; Gatesoupe, 1999). By supplying probiotics via culture water a constant, uniform presence could be maintained within the animal's G.I. tract. The link between microbial activity, water quality and disease control in aquaculture systems is well established (Ninawe & Selvin, 2009). The effects of such microfloral management would likely be magnified in hatchery and nursery facilities where larval and post-larval animals display the greatest mortality due to bacterial pathogens (Nogami & Maeda, 1992; Garriques & Arevalo, 1995). Indeed, the different host/microbiota interactions in aquatic animals have led many scientists to advocate the expansion of the definition of probiotic to encompass the addition of autochthonous bacteria to tanks and ponds (Moriarty, 1999; Verschuere et al, 2000; Ninawe & Selvin, 2009). Undeniably it makes sense to have separate definitions of what comprises terrestrial and aquatic probiotics, due to the radical differences in the natures of the two environments.

In conclusion, probiotic bacteria used in conjunction with effective non-microbial immunostimulants and improved farm management techniques (e.g. polyculture and use of lined ponds) could form an extremely viable, cost effective and environmentally sound means of controlling disease (and effluent) in commercial penaeid aquaculture (Dierberg & Kiattisimkul, 1996). This has been reflected in the amount of research into the efficacy of numerous species of bacteria as shrimp probiotics published in the last two years (Li et al, 2007; Vieira et al, 2007; Chiu et al, 2007; Balcázar et al, 2007; Balcázar & Rojas-Luna, 2007; Castex et al, 2008; Gómez & Shen, 2008). However, probiotics should not be considered a 'silver bullet'; even an optimally managed facility, correctly utilising probiotics, immunostimulants and therapeutic anti-microbials will still be susceptible to disease outbreaks, such is the

nature of intensive aquaculture. It has been hypothesised (and widely accepted) that many of the *Vibrio* spp. associated with shrimp disease are opportunistic pathogens and only cause mass mortality when animals are in poor condition or stressed (Lightner, 1993; Ruangpan & Kitao, 1991). Consequently, a well managed facility with a diverse microflora (not overtly dominated by the Vibrionaceae) should experience a vastly reduced incidence of disease outbreaks. Considerable further investigation is required to increase the level of understanding of what constitutes healthy penaeid microflora. In particular the use of molecular techniques in examining microbial diversity/composition at the community level is important given that many marine bacterial endosymbionts are unculturable on available media (Zengler et al, 2002: Sakami et al, 2008).

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(http://news.bbc.co.uk/2009)

Appendices

Appendix 1

Marine anticoagulant

Sodium chloride	(NaCl)	26.3 g
Glucose	($C_6H_{12}O_6$)	19.0 g
Trisodium citrate	($Na_3C_6H_5O_7.2H_2O$)	8.8 g
Citric acid	($C_6H_8O_7.H_2O$)	5.4 g
EDTA	($C_{10}H_{16}N_2O_8$)	3.7 g
Distilled deionised H_2O		1 L

Shrimp marine anticoagulant

Trisodium citrate	($Na_3C_6H_5O_7.2H_2O$)	8.8 g
Sodium chloride	(NaCl)	19.8 g
EDTA	($C_{10}H_{16}N_2O_8$)	3.0 g
Glucose	($C_6H_{12}O_6$)	1.1 g
Distilled deionised H_2O		1 L

Isosmotic formalin solution

Formalin	(40% by volume CH_2O)	250 ml
Glucose	($C_6H_{12}O_6$)	1.1 g
Distilled deionised H_2O		750 ml

(Sodium) Cacodylate buffer

Sodium chloride	(NaCl)	1.3 g
Sodium cacodylate	($(CH_3)_2AsNaO_2.3H_2O$)	1.0 g
Trisodium citrate	($Na_3C_6H_5O_7.2H_2O$)	11.0 g
Distilled deionised H_2O		500 ml

Cacodylate sucrose buffer

Sodium cacodylate	($(CH_3)_2AsNaO_2.3H_2O$)	1.3 g
Sucrose	($C_{12}H_{22}O_{11}$)	43.0 g
Calcium chloride	($CaCl_2.2H_2O$)	7.5 g
Distilled deionised H_2O		500 ml

L-Dopa solution

L-β-3,4-Dihydroxyphenylalanine	($C_9H_{11}NO_4$)	3 mg
MilliQ water		1 ml

Marine saline solution

Sodium chloride	(NaCl)	33.7 g
Potassium chloride	(KCl)	1.0 g
Calcium chloride	($CaCl_2$)	3.0 g
Tris (hydroxymethyl)-methylamine	($C_4H_{11}NO_3$)	6.0 g
Sodium dihydrogen orthophosphate	($NaH_2PO_4.2H_2O$)	0.1 g
MilliQ water		1 L
(Attenuated to pH 7.4)		

Seawater Bouin's fixative

Seawater saturated picric acid	($C_6H_3N_3O_7$)	75 ml
Formalin	(40% by volume CH_2O)	25 ml
Glacial acetic acid	($C_2H_4O_2$)	5 ml

Cole's haematoxylin (Humason)

1% iodine in 95% ethanol	(I in C_2H_5OH)	2 ml
10% haematoxylin in 100% ethanol	($C_{16}H_{14}O_6$ in C_2H_5OH)	11 ml
1.2% potassium aluminium sulphate	($KAl(SO_4)2.12H_2O$)	

Alcoholic eosin

Eosin		0.1 g
70% ethanol	(C_2H_5OH)	100 ml
Glacial acetic acid	($C_2H_4O_2$)	0.5 ml

Tris-borate-EDTA (TBE) buffer

Tris (hydroxymethyl)-methylamine	($C_4H_{11}NO_3$)	54.0 g
Boric acid	(H_3BO_3)	27.5 g
Distilled deionised H_2O		900 ml
0.5 M EDTA	($C_{10}H_{16}N_2O_8$)	20 ml
Distilled deionised H_2O		80 ml

Formula for the calculation of phenoloxidase (PO) specific activity:

Result of the assay, i.e. PO specific activity, is expressed as units of absorbance at 492nm.per min. per mg of protein (@492/min/mg protein) and is calculated thus;

$$\text{Specific Activity (SA)} = \frac{\text{Abs. at 492nm x dilution factor}}{\text{([Protein] of HLS (mg/ml) x Time incubated x Volume of proPO extract (ml))}}$$

$$\text{Dilution factor} = \frac{\text{HLS + Trypsin + L-Dopa}}{\text{HLS + Trypsin + L-Dopa}} = 1$$

Appendix 2

Table displaying the number of viable *Lactobacillus plantarum* colonies recovered from crab feed

Lab Ref.	Product Name	Cust. Ref.	Date Recd.	Date Tst.	TVC	LAB	Comments
6980	Plantarum mix (crab feed)	99028S2/99033S2/98031S1	na	28/11/2005	nt	1.58E+11	
6981	Crab feed (1% plantarum)		28/11/2005	28/11/2005	6.00E+03	8.20E+07	Not stomached - just whirlimixed
6982	Crab feed (control)		28/11/2005	28/11/2005	6.00E+04	<1x10e3/g	Not stomached - just whirlimixed
7066	Crab feed (probiotic)	5/12/05 w2	05/12/2005	06/12/2005	nt	6.70E+07	
7067	Crab feed (control)	5/12/05 w2	05/12/2005	06/12/2005	5.00E+08	nt	
7086	Crab feed (probiotic)	12/12/05 T3	12/12/2005	12/12/2005	5.20E+07	5.00E+07	TVC - all one type (presumably plantarum)
7087	Crab feed (control)	12/12/05 T3	12/12/2005	12/12/2005	2.40E+07	nt	TVC - general mixture of bacteria

All were tested by putting 1g in 90ml MRD (except 6980). Samples received were approx 2g jelly-like rectangular blocks

Appendix 3

Table of bacterial isolates derived from the Pacific white shrimp *L. vannamei* tested against potential shrimp pathogens; no interference/inhibition (-), growth interference/inhibition observed (+), possible interference (P). Marine agar (MA), Tryptic soy agar plus 2% NaCl (TSA).

Isolate I.D.	Method of Isolation & Source	Media & Temp	Antagonistic activity against pathogens					
			Cell free supernatant			Pathogen-isolate *in vitro* co-culture		
			V. harveyi	*L. anguillarum*	*V. alginolyticus*	*V. harveyi*	*L. anguillarum*	*V. alginolyticus*
L1	Spread plating/post larvae [whole]	MA [15°C]	-	-	-	-	-	-
L1 - 1	Spread plating/post larvae [whole]	TSA [25°C]	-	-	-	-	-	-
L1 - 2	Spread plating/post larvae [whole]	TSA [25°C]	-	-	-	-	-	-
L1 - 3	Spread plating/post larvae [whole]	MA [25°C]	-	-	-	-	-	-
L1 - 4	Spread plating/post larvae [whole]	TSA [25°C]	-	-	-	-	-	-
L1 - 5	Spread plating/post larvae [whole]	TSA [25°C]	-	-	-	-	-	-
L1 - 6	Spread plating/post larvae [whole]	TSA [25°C]	-	-	-	-	-	-
L1 - 9	Spread plating/post larvae [whole]	TSA [25°C]	-	-	-	-	-	-
L2	Spread plating/post larvae [whole]	MA [25°C]	-	-	-	-	-	-
L2 - 2	Spread plating/post larvae [whole]	TSA [25°C]	-	-	-	-	-	-
L2 - 7	Spread plating/post larvae [whole]	TSA [25°C]	-	-	-	-	-	-
L2 - 8	Spread plating/post larvae [whole]	TSA [25°C]	-	-	-	-	-	-
L2 - 9	Spread plating/post larvae [whole]	TSA [25°C]	-	-	-	-	-	-
L3	Spread plating/post larvae [whole]	MA [25°C]	-	-	-	-	-	-
10L1	Spread plating/post larvae [whole]	MA + PO_4 salt [25°C]	-	-	-	-	-	-
10L2	Spread plating/post larvae [whole]	MA + PO_4 salt [25°C]	-	-	-	-	-	-
G1	Spread plating/post larvae [gut]	MA [25°C]	-	-	-	-	-	-
G2	Spread plating/post larvae [gut]	MA [25°C]	-	-	-	-	-	-
G3	Spread plating/post larvae [gut]	MA [25°C]	-	-	-	-	-	-
G3 - 1	Spread plating/post larvae [gut]	MA [15°C]	-	-	-	-	-	-
G4	Spread plating/post larvae [gut]	MA [25°C]	-	-	-	-	-	-
G4 - 1	Spread plating/post larvae [gut]	TSA [25°C]	-	-	-	-	-	-
G4 - 2	Spread plating/post larvae [gut]	TSA [25°C]	-	-	-	-	-	-

Table continued on next pag

G5	Spread plating/post larvae [gut]	MA [25°C]	-	-	-	-	-	-
G5 - 1	Spread plating/post larvae [gut]	TSA [25°C]	-	-	-	-	-	-
G6	Spread plating/post larvae [gut]	MA [25°C]	-	-	-	-	-	-
G6 - 1	Spread plating/post larvae [gut]	TSA [25°C]	-	-	-	-	-	-
G7	Spread plating/post larvae [gut]	TSA [25°C]	-	-	-	-	-	-
G7 - 1	Spread plating/post larvae [gut]	TSA [25°C]	-	-	-	-	-	-
G7 - 2	Spread plating/post larvae [gut]	TSA [25°C]	-	-	-	-	-	-
10G1	Spread plating/post larvae [gut]	MA + PO_4 salt [25°C]	-	-	-	-	-	-
10G2	Spread plating/post larvae [gut]	MA + PO_4 salt [25°C]	-	-	-	-	-	-
10G3	Spread plating/post larvae [gut]	MA + PO_4 salt [25°C]	-	-	-	-	-	-
10G4	Spread plating/post larvae [gut]	MA + PO_4 salt [25°C]	-	-	-	-	-	-
10G5	Spread plating/post larvae [gut]	MA + PO_4 salt [25°C]	-	-	-	-	-	-
10G6	Spread plating/post larvae [gut]	MA + PO_4 salt [25°C]	-	-	-	-	-	-
C-G2	Spread plating/post larvae [gut]	MA [25°C]	-	-	-	-	-	-
HP1	Spread plating/post larvae [Hepato]	MA [25°C]	-	-	-	-	-	-
HP1 - 1	Spread plating/post larvae [Hepato]	TSA [25°C]	-	-	-	-	-	-
HP2	Spread plating/post larvae [Hepato]	MA [25°C]	-	-	-	-	-	-
HP2 - 1	Spread plating/post larvae [Hepato]	MA [15°C]	-	-	-	-	-	-
HP4	Spread plating/post larvae [Hepato]	MA [25°C]	-	-	-	-	-	-
HP5	Spread plating/post larvae [Hepato]	MA [25°C]	-	-	-	-	-	-
HP6	Spread plating/post larvae [Hepato]	MA [25°C]	-	-	-	-	-	-
HP8	Spread plating/post larvae [Hepato]	TSA [25°C]	-	-	-	-	-	-
HP13	Spread plating/post larvae [Hepato]	TSA [25°C]	-	-	-	-	-	-
HP14	Spread plating/post larvae [Hepato]	TSA [25°C]	-	-	-	-	-	-
100HP1	Spread plating/post larvae [Hepato]	MA + PO_4 salt [25°C]	-	-	-	-	-	-
C-HP2	Spread plating/post larvae [Hepato]	MA [25°C]	-	-	-	-	-	-
C-HP3	Spread plating/post larvae [Hepato]	MA [25°C]	-	-	-	-	-	-
G-A1	Spread plating/adult [gut]	TSA [25°C]	-	-	-	-	-	-

Table continued on next pag

G-A2	Spread plating/adult [gut]	TSA [25°C]	-	-	-	-	-	-
G-A3	Spread plating/adult [gut]	TSA [25°C]	-	-	-	-	-	-
G-A4	Spread plating/adult [gut]	TSA [25°C]	-	-	-	-	-	-
G-A5	Spread plating/adult [gut]	TSA [25°C]	-	-	-	-	-	-
G-A6	Spread plating/adult [gut]	TSA [25°C]	-	-	-	-	-	-
G-A7	Spread plating/adult [gut]	TSA [25°C]	-	-	-	-	-	-
G-A8	Spread plating/adult [gut]	TSA [25°C]	-	-	-	-	-	-
G-A9	Spread plating/adult [gut]	TSA [25°C]	-	-	-	-	-	-
G-A10	Spread plating/adult [gut]	TSA [25°C]	-	-	-	-	-	-
G-A11	Spread plating/adult [gut]	TSA [25°C]	-	-	-	-	-	-
G-A12	Spread plating/adult [gut]	TSA [25°C]	-	-	-	-	-	-
G-A13	Spread plating/adult [gut]	TSA [25°C]	-	-	-	-	-	-
G-A14	Spread plating/adult [gut]	TSA [25°C]	-	-	-	-	-	-
G-B	Spread plating/adult [gut]	MRS [37°C]	+	-	+		Incompatible media	
HP-A1	Spread plating/adult [Hepato]	TSA [25°C]	-	-	-	-	-	-
HP-A2	Spread plating/adult [Hepato]	TSA [25°C]	-	-	-	-	-	-
HP-A3	Spread plating/adult [Hepato]	TSA [25°C]	-	-	-	-	-	-
HP-A4	Spread plating/adult [Hepato]	TSA [25°C]	-	-	-	-	-	-
HP-A5	Spread plating/adult [Hepato]	MA [25°C]	-	-	-	-	-	-
HP-A6	Spread plating/adult [Hepato]	MA [25°C]	-	-	-	-	-	-
HP-A7	Spread plating/adult [Hepato]	TSA [25°C]	-	-	-	-	-	-
HP-A8	Spread plating/adult [Hepato]	TSA [25°C]	-	-	-	-	-	-
HP-A9	Spread plating/adult [Hepato]	TSA [25°C]	-	-	-	-	-	-
HP-A10	Spread plating/adult [Hepato]	TSA [25°C]	-	-	-	-	-	-
HP-A11	Spread plating/adult [Hepato]	TSA [25°C]	-	-	-	-	-	-
HP-A12	Spread plating/adult [Hepato]	MA [25°C]	-	-	-	-	-	-
HP-A13	Spread plating/adult [Hepato]	MA [25°C]	-	-	-	-	-	-
HP-A14	Spread plating/adult [Hepato]	MA [25°C]	-	-	-	-	-	-
HP-A15	Spread plating/adult [Hepato]	MA [25°C]	-	-	-	-	-	-

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HP-A16	Spread plating/adult [Hepato]	MA [25°C]	-	-	-	-	-	-
HP-A17	Spread plating/adult [Hepato]	MA [25°C]	-	-	-	-	-	-
HP-A18	Spread plating/adult [Hepato]	MA [25°C]	-	-	-	-	-	-
HP-A19	Spread plating/adult [Hepato]	MA [25°C]	-	-	-	-	-	-
HP-A20	Spread plating/adult [Hepato]	MA [25°C]	-	-	-	-	-	-
HP-B	Spread plating/adult [Hepato]	MRS [37°C]	-	-	-		Incompatible media	
HP-B1	Spread plating/adult [Hepato]	MRS [37°C]	+	-	+		Incompatible media	
G1-I1	Nitrocellulose *in vitro* co-culturing/adult [gut]	TSA [25°C]				-	-	-
G1-I2	Nitrocellulose *in vitro* co-culturing/adult [gut]	TSA [25°C]				-	-	-
G1-I3	Nitrocellulose *in vitro* co-culturing/adult [gut]	TSA [25°C]				P	-	P
G1-I4	Nitrocellulose *in vitro* co-culturing/adult [gut]	TSA [25°C]				-	-	-
G1-I5	Nitrocellulose *in vitro* co-culturing/adult [gut]	TSA [25°C]				-	-	-
G1-I6	Nitrocellulose *in vitro* co-culturing/adult [gut]	TSA [25°C]				-	-	-
G1-I7	Nitrocellulose *in vitro* co-culturing/adult [gut]	TSA [25°C]				P	-	-
G2-I1	Nitrocellulose *in vitro* co-culturing/adult [gut]	TSA [25°C]				-	-	-
G2-I2	Nitrocellulose *in vitro* co-culturing/adult [gut]	TSA [25°C]				-	-	-
G2-I3	Nitrocellulose *in vitro* co-culturing/adult [gut]	TSA [25°C]				-	-	-
G2-I4	Nitrocellulose *in vitro* co-culturing/adult [gut]	TSA [25°C]				-	-	-
G2-I5	Nitrocellulose *in vitro* co-culturing/adult [gut]	TSA [25°C]				-	-	-
G2-I6	Nitrocellulose *in vitro* co-culturing/adult [gut]	TSA [25°C]				-	-	-
I1	Spread plating/adult [gut]	MRS [37°C]	-	-	-	-	-	-
I2	Spread plating/adult [gut]	MRS [37°C]	-	-	-	-	-	-
I3	Spread plating/adult [gut]	MRS [37°C]	-	-	-	-	-	-
I4	Spread plating/post larvae [Hepato]	MRS [37°C]	-	-	-	-	-	-
I5-A	Spread plating/adult [gut]	MRS [37°C]	-	-	-	-	-	-
I5-B	Spread plating/adult [gut]	MRS [37°C]	-	-	-	-	-	-
I6	Spread plating/adult [gut]	MRS [37°C]	-	-	-	-	-	-
I7	Spread plating/adult [gut]	MRS [37°C]	-	-	-	-	-	-
I8	Spread plating/adult [gut]	MRS [37°C]	-	-	-	-	-	-

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I9	Spread plating/adult [gut]	MRS [37°C]	-	-	-	-	-	-
I10	Spread plating/adult [gut]	MRS [37°C]	-	-	-	-	-	-
I11	Spread plating/adult [gut]	MRS [37°C]	-	-	-	-	-	-
I12	Spread plating/adult [gut]	MRS [37°C]	-	-	-	-	-	-
I13	Spread plating/tank [Biofilm]	MRS [37°C]	-	-	-	-	-	-

Isolate I.D.	Gram (+ or -)	Cell morphology	Size (µm)		Gross colony morphology		
			Length	Width	Form	Margin	Elevation
L1	-	Short rod	1.8	1.2	Circular	Entire	Raised
L1 - 1	-	Coccoid	1.2	-	Spreader	Undulate	Flat
L1 - 2	-	Short rod	1.2	1.8	Circular	Entire	Raised
L1 - 3	-	Rod	1.8	0.6	Circular	Entire	Raised
L1 - 4	-	Rod	1.8	0.8	Circular	Entire	Flat
L1 - 5	-	Coccoid	1.2	-	Circular	Entire	Raised
L1 - 6	-	Coccoid	1.8	-	Circular	Entire	Convex
L1 - 9	-	Short rod	1.6	1.2	Circular	Entire	Convex
L2	-	Short rod	2.0	1.2	Circular	Entire	Raised
L2 - 2	-	Short rod	1.2	1.8	Spreader	Undulate	Flat
L2 - 7	-	Coccoid	1.2	-	Circular	Entire	Convex
L2 - 8	-	Short rod	1.5	1.0	Circular	Entire	Convex
L2 - 9	-	Rod	2.4	1.0	Circular	Entire	Convex
L3	-	Short rod	1.8	1.0	Circular	Entire	Raised
10L1	-	Coccoid	1.0	-	Circular	Undulate	Flat
10L2	+	Coccoid	0.6	-	Circular	Entire	Pulvinate
G1	-	Rod	2.4	0.6	Circular	Entire	Convex
G2	-	Rod	2.4	0.6	Circular	Entire	Convex
G3	-	Rod	3.6	1.2	Circular	Entire	Convex
G3 - 1	-	Rod	2.4	1.0	Circular	Entire	Convex
G4	-	Rod	1.8	0.4	Circular	Entire	Convex
G4 - 1	-	Coccoid	1.2	-	Circular	Entire	Convex
G4 - 2	-	Coccoid	1.2	-	Circular	Entire	Convex
G5	-	Rod	2.4	0.8	Circular	Entire	Convex
G5 - 1	-	Rod	1.8	0.8	Circular	Entire	Convex
G6	-	Rod	2.4	1.2	Circular	Entire	Raised
G6 - 1	-	Short rod	1.5	0.6	Circular	Entire	Convex
G7	-	Short rod	1.2	1.8	Circular	Entire	Convex
G7 - 1	-	Rod	2.4	1.0	Circular	Entire	Convex
G7 - 2	-	Coccoid	1.2	-	Circular	Entire	Convex
10G1	-	Rod	1.8	0.4	Circular	Entire	Flat
10G2	-	Coccoid	1.2	-	Circular	Undulate	Pulvinate
10G3	-	Rod	3.0	1.2	Circular	Entire	Convex
10G4	-	Thin rod	2.4	0.5	Circular	Entire	Raised
10G5	-	Rod	2.4	1.2	Circular	Undulate	Convex
10G6	-	Thin rod	2.4	0.5	Circular	Entire	Raised
C-G2	-	Rod	1.2	0.3	Circular	Undulate	Flat
HP1	-	Rod	1.2	0.4	Circular	Entire	Raised
HP1 - 1	-	Coccoid	1.2	-	Circular	Entire	Convex
HP2	-	Rod	1.2	0.6	Circular	Entire	Raised
HP2 - 1	-	Short rod	1.8	0.8	Circular	Entire	Raised
HP4	-	Rod	1.5	0.6	Circular	Entire	Raised
HP5	-	Short rod	1.8	0.8	Circular	Entire	Raised
HP6	-	Short rod	1.2	0.4	Circular	Entire	Raised
HP8	-	Coccoid	0.6	-	Circular	Entire	Convex
HP13	-	Spherical	1.2	-	Circular	Entire	Convex
HP14	-	Short rod	1.5	1.0	Circular	Entire	Convex
100HP1	-	Short rod	2.2	1.4	Circular	Undulate	Flat
C-HP2	-	Coccoid	1.2	-	Irregular	Undulate	Raised
C-HP3	+	Coccoid	0.6	-	Circular	Entire	Convex