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Protection of gnotobiotic *Artemia* against *Vibrio campbellii* using baker's yeast strains and extracts

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences (Aquaculture)

Dutch translation of the title: “Bescherming van gnotobiotische *Artemia* tegen *Vibrio campbellii* door bakkersgiststammen en –extracten”

Cover picture: Epifluoresence microphotographs of *Artemia* loaded with fluorescent bacteria

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List of abbreviations and units

°C	Degree Celsius
%	Percentage
$\mu Em^{-2}s$	Micro Einstein per second and square meter
µg	Microgram
µm	Micrometre
µl	Microlitre
λ	Wavelength
γ	Gamma
× g	Relative centrifugal force or G force
±	Approximately
/	Per
kDa	KiloDalton
AFDW	Ash free dry weight
AML1	Acute Myeloid Leukemia 1
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
APC	Antigen presenting cells
apoE	apolipoprotein E
apoLp-III	Apolipophorin III
ARC	Artemia Reference Center
βGRP	β-glucan recognition proteins
bp	base pair
BRM	Biological response modifiers
BSE	Bovine spongiform encephalopathy
C3	Complement type 3
CARD	Caspase-recruitment domain
cDNA	Complementary deoxyribonucleic acid
chs3	Mutant with a deleted gene involved in chitin biosynthesis (capital letters indicate the gene involved)
CLEC7A	C-type lectin domain family 7, member A
cm	Centimetre
CR3	Complement receptor type 3
CRD	Carbohydrate recognition domain
CTL	C-type lectin
DC	Dendritic cell

DC-SIGN	A novel DC-specific C-type lectin, which is abundantly expressed by dendritic cells both in vitro and in vivo, mediates transient adhesion with T cells.
Dif factor	A sequence-specific transcription factor and is probably a key activator of the immune response in *Drosophila*
Dorsal factor	Dorsal is the focal protein activating and repressing zygotic genes responsible for differentiation along the dorsoventral axis during the early stages of flies development
DP	Degree of polymerization
DNA	Deoxyribonucleic acid
dSRCI	*Drosophila* scavenger receptor
dTAK1	*Drosophila* transforming growth factor activated kinase 1 gene
DW	Dry weight
EU	European Union
EUROSCARF	European *Saccharomyces cerevisiae* Archive for Functional Analysis, Germany
exp	Exponential growth phase
Exp …	Experiment number …
FAO	Food and Agricultural Organization of the United Nations
FASW	Filtered and autoclaved seawater
fks1	Mutant with a deleted gene involved in β-1,3 glucan biosynthesis (capital letters indicate the gene involved)
FT	Falcon tube
g	Gram
GART	Gnotobiotic *Artemia* test
gas1	Mutant with a deleted gene involved in lycosylphosphatidylinositol (GPI) protein (capital letters indicate the gene involved)
GATA-1	A key regulator of erythroid-cell differentiation and survival in mammals
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GNBPs	Gram-negative binding proteins
GBP	Glucan binding protein
GPI	Glycosylphosphatidylinositol
Gcm	Glial cell missing gene
GCs	Granular cells
GRAS	Generally regarded as safe
h	Hour
HC	Hyaline cell
hDectin-1	Human Dectin-1
Hd	*Holotrichia diomphalia*
HDL	High density lipoproteins
HPAEC	High performance anionic exchange chromatography
hpt	hematopoietic tissue

HSP	Heat shock proteins
iC3b	inactive form of C3b
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon-gamma
Iκ-B	Inhibitor kappa B
IKK	Inhibitor of kappa B protein−kinase
IL	Individual length
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-12	Interleukin 12
Imd/relish	A second immune signalling pathway in *Drosophila* immunity involved in defence against Gram-negative bacteria
IRAK	IL-1 receptor associated kinases
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JAK/STAT	An immune pathway in Drosophila
kg	Kilogram
knr4	Mutant with a deleted gene involved in β-1,3 glucan biosynthesis (capital letters indicate the gene involved)
kre6	Mutant with a deleted gene involved in β-1,6 glucan biosynthesis (capital letters indicate the gene involved)
l	litre
LacCer	Lactosylceramide
LDL	Low density lipoproteines
LMG	Laboratory of Microbiology of the Ghent University (Culture Collection)
LPS	Lipopolysaccharide
LVS…	Strain … isolated by Laurent Verschuere
Lyn kinase	A protein which plays a key role in the growth and apoptotic regulation of hematopoietic cells in mamals
lz	lozenge gene
M	Molar
MA	Marine agar 2216
MAPKKK	Mammalian mitogen-activated protein kinase kinase kinase
Mac-1	Membrane associated component 1
med	Medium
2ME	2-mercapto-ethanol
mg	Milligram
Microarray	A method for profiling gene and protein expression in cells and tissues
min	Minute

MIP	Macrophage inflammatory protein
ml	Millilitre
mm	Millimetre
mnn…	Mutants with deleted genes involved in the extension of mannose in cell wall proteins (capital letters indicate the gene involved) (see details in Chapter III)
MPO	Myeloperoxidase
MTT	-3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide
MW	Molecular weigth
MyD88	Myeloid differentiation factor 88
n	Number of replicates
NADP	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappa B
NK cells	Natural killer cells
NMR	Nuclear magnetic resonance
OD	Optical density
OD…	Optical density at … nm
ORF	Open reading frame
p	Statistical p-value obtained
PA	Phenoloxidase activating enzyme
PAMP	Pathogen associated molecular patterns
PBMC	Proliferation of peripheral blood mononuclear cells
PGE_2	Prostaglandin E2
PGN	Peptidoglycan
PGRPs	Peptidoglycan recognition proteins
PGRP-LC	*Drosophila* peptidoglycan recognition protein LC
pH	Measure of the acidity of a solution
PlRunt	A gene encoding a Runt protein
ppt	Part per thousand
PO	Phenoloxidase
PPO	Prophenol oxidase
proPO	prophenoloxidase
PRRs	Pattern recognition receptors
PVF2/PVR	An immune pathway in *Drosophila*
Ras/Raf	An immune pathway in *Drosophila*
Rel/NF-kB	A collection of proteins, conserved from the fruit fly *Drosophila melanogaster* to humans, involved in the control of a large number of normal cellular and organismal processes, such as immune and inflammatory responses, developmental processes, cellular growth, and apoptosis
RNA	Ribonucleic acid

ROS	Reactive oxygen species
rpm	Rotations per minute
rRNA	Ribosomal ribonucleic acid
s	Second
SCP	Single Cell Proteins
SPG	Shizophyllan
SRs	Scavenger receptors
SR-CI	Scavenger receptor class C
SD	Standard deviation
SGCs	Semi-granular cells
SIGNR1	Murine homologue of DC-SIGN
srp	serpent gene
stat	Stationary growth phase
TBP	Total biomass production
Th	T helper cell
TLRs	Toll-like receptors
TN	Treatment number
TNF	Tumur necrosis factor
TRAF	TNF receptor associated factor
USA	United States of America
Ush	U-shaped gene
US$	United States of America dollars
v/v	Volume per volume
VC	*Vibrio campbellii*
VP	*Vibrio proteolyticus*
w/v	Weight per volume
WT	Wild type yeast strain
YEPD	Yeast extract peptone dextrose medium
YNB	Yeast nitrogen based medium
ZEN	Zearalenone

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CHAPTER I

INTRODUCTION

Introduction

I. Importance of aquaculture

Capture fisheries and aquaculture supplied the world with about 106 million tonnes of food fish in 2004, providing an apparent per capita supply of 16.6 kg (live weight equivalent), which is the highest on record (Table 1.1 and Fig. 1.1) (FAO, 2006). Of this supply, aquaculture accounted for 43 percent. The contribution of aquaculture to global supplies of fish, crustaceans, molluscs and other aquatic animals continues to grow, increasing from 3.9 percent of total production by weight in 1970 over 27.1 percent in 2000 and 32.4 percent in 2004 (FAO, 2006). Aquaculture production in terms of quantity and value for major species groups in 2004 is presented in Fig. 1.2.

Aquaculture continues to grow more rapidly than any other animal food-producing sectors. Worldwide, the sector has grown at an average rate of 8.8 percent per year since 1970, compared with only 1.2 percent for capture fisheries and 2.8 percent for terrestrial farmed meat production systems over the same period. As capture fisheries are expected to stagnate, the increasing demand for aquatic products will have to be provided by aquaculture. The per capita supply from aquaculture increased from 0.7 kg in 1970 to 7.1 kg in 2004.

At the global animal production level, animal disease outbreaks could represent an important source of uncertainty. For example, during the past few years, and particularly in 2004 and 2005, the international market for meats was disrupted by outbreaks of animal diseases such as avian influenza and bovine spongiform encephalopathy (BSE). This situation, together with the related import bans, led to an inducted shortage in meat supplies in some countries, particularly of poultry, pushing up international meat prices in 2004 and 2005 (+30 percent for poultry in 2004–05) and driving consumers towards alternative protein sources, including fish.

World aquaculture (food fish and aquatic plants) has grown significantly during the past half-century. From a production of below 1 million tonnes in the early 1950s production in 2004 was reported to have risen to 59.4 million tonnes, with a value of US$ 70.3 billion. This represents an average annual increase of 6.9 percent in quantity and 7.7 percent in value over reported figures for

2002. In 2004, countries in the Asia and the Pacific region accounted for 91.5 percent of the production quantity and 80.5 percent of the value. Of the world total, China is reported to account for 69.6 percent of the total quantity and 51.2 percent of the total value of aquaculture production (see below).

Table 1.1- World fisheries and aquaculture production and utilization (adapted from FAO, 2006)

	2000	2001	2002	2003	2004	2005
			(Million tonnes)			
PRODUCTION						
INLAND						
Capture	8.8	8.9	8.8	9.0	9.2	9.6
Aquaculture	21.2	22.5	23.9	25.4	27.2	28.9
Total inland	30.0	31.4	32.7	34.4	36.4	38.5
MARINE						
Capture	86.8	84.2	84.5	81.5	85.8	84.2
Aquaculture	14.3	15.4	16.5	17.3	18.3	18.9
Total marine	101.1	99.6	101.0	98.8	104.1	103.1
TOTAL CAPTURE	95.6	93.1	93.3	90.5	95.0	93.8
TOTAL AQUACULTURE	35.5	37.9	40.4	42.7	45.5	47.8
TOTAL WORLD FISHERIES	131.1	131.0	133.7	133.2	140.5	141.6
UTILIZATION						
Human consumption	96.9	99.7	100.2	102.7	105.6	107.2
Non-food uses	34.2	31.3	33.5	30.5	34.8	34.4
Population *(billions)*	6.1	6.1	6.2	6.3	6.4	6.5
Per capita food fish supply *(kg)*	16.0	16.2	16.1	16.3	16.6	16.6

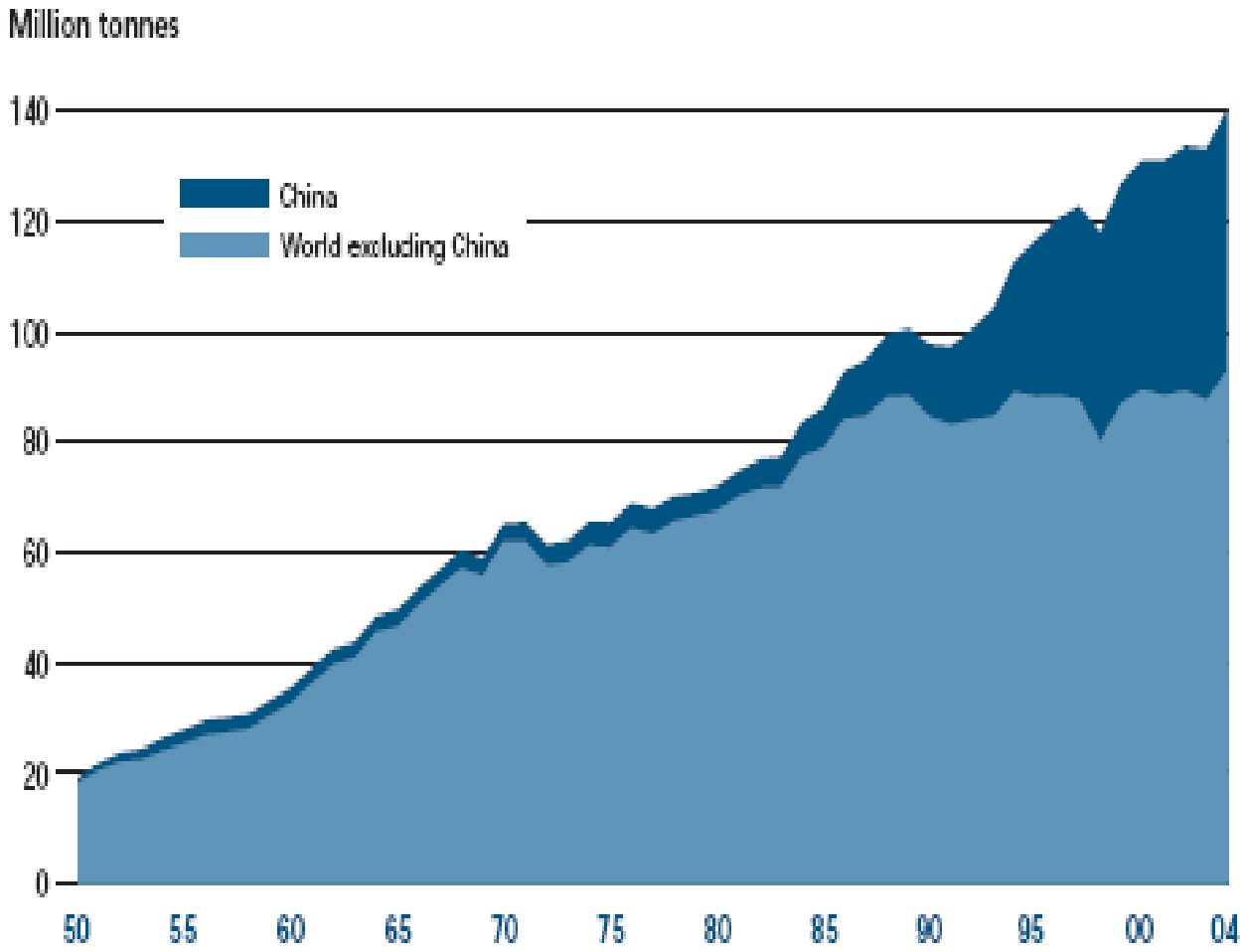

Fig.1.1- World capture and aquaculture production (adapted from FAO, 2006)

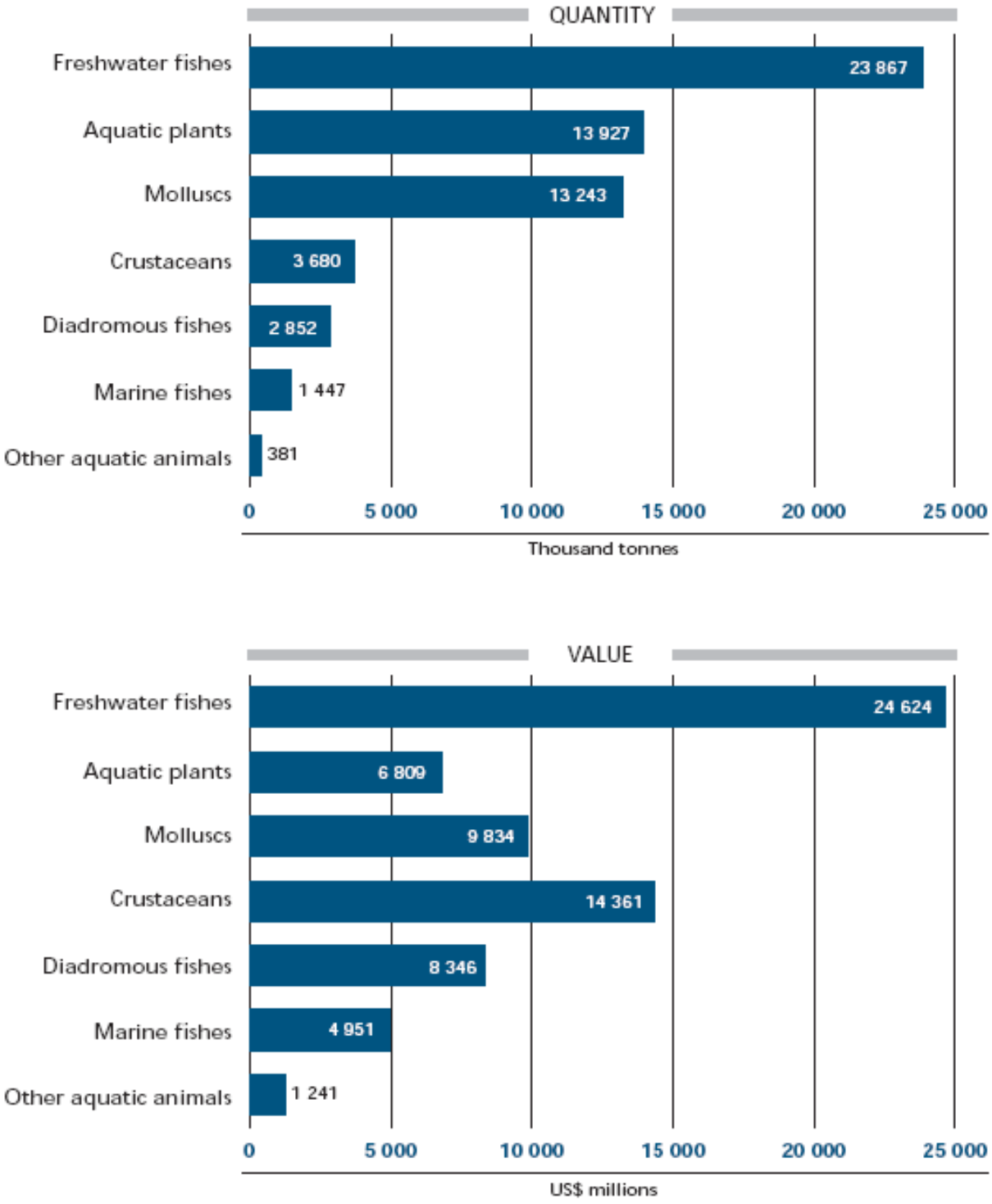

Fig.1.2- World aquaculture production by major species groups in 2004 (After FAO, 2006).

II. Diseases control in aquaculture

Diseases are still the major constraint of aquaculture production and trade (FAO, 2004) causing unpredictable massive mortalities especially in the early life stages of aquatic organisms (mainly for

marine fish and shellfish species) as a result of the proliferation of pathogenic and opportunistic microorganisms in the culture systems. The trade and movement of live aquatic organisms has increased the worldwide translocation of pathogens resulting in a fast spread of diseases. Several solutions have been applied to control diseases in aquaculture.

Many chemotherapeutics, such as antibiotics, drugs and other pharmacologically active compounds, either in the culture water or in the fish feed (Touraki *et al.*, 1999; Vaseeharan *et al.*, 2004) have been used. These antimicrobial drugs contributed to the development of aquaculture. They were administered not only as therapeutic but also as preventive solutions to avoid diseases. However, extreme use of antibiotics can cause several environmental disorders (*e.g.* contamination of the culture environment, damage to organisms, and creation of bacterial resistance that can be spread in the food chain (Kautsky *et al*., 2000) as well as human health disorders (*e.g.* allergies, toxic effect, changing the microflora of gastrointestinal tract and production of antimicrobial-resistant pathogenic bacteria).

Although antibiotics are still used in some hatcheries, their application is gradually banned or restricted due to severe negative side effects.

Nowadays, several alternative and environmental-friendly prophylactic and preventive methods are being developed to control diseases and to maintain a healthy microbial environment in aquaculture systems.

One approach is the improvement of the larval quality through better nutrition, focusing on beneficial effects of some nutrients, such as unsaturated fatty acids, sterols, proteins, carbohydrates, trace elements and vitamins (Lall, 2000; Soudant *et al.*, 2000; Lavens and Sorgeloos, 2000; Chen *et al.*, 2005). It is well known that nutritional and physical characteristics of diets can modulate susceptibility of an organism to infectious diseases (Lall, 2000). Another approach currently gaining acceptance within the aquaculture industry is the use of probiotic microorganisms as a prophylactic and preventive solution against diseases.

There is increasing evidence that microflora manipulation, or addition of probionts, may improve health conditions, enhance larval survival and prevent the proliferation and colonization of opportunistic and/or pathogenic bacteria in intensive rearing systems (Gatesoupe, 1999; Verschuere *et al.*, 2000a; Villamil *et al.*, 2003; Gullian *et al*., 2004; Vázquez *et al.*, 2005). However, the results obtained in laboratory conditions are sometimes different from those observed in the field. This limitation can eventually be lifted by studying in detail the host-microbial interactions.

The use of microbially matured water is another technique that is applied in aquaculture. In fact, microbial maturation of seawater with a biofilter or water from previously well-performing cultures can be useful tools to control the microbiota, protecting marine larvae from the proliferation of detrimental opportunistic bacteria, eventually resulting in enhanced larval performance. (Skjermo *et al.,* 1997).

Green water technique is another tool, which is based on the addition of microalgae in closed water systems under low light-intensity conditions that do not allow their proliferation (Papandroulakis *et al.*, 2001). Several studies indicate that some microalgae and cyanobacteria provide beneficial effects to the cultured organisms (Cahu *et al.,* 1998; Cohen, 1999; Salvesen *et al*., 2000; Pulz and Gross, 2004).

The induction of Heat Shock Protein (HSP) expression can activate the immune system of an organism resulting in increased tolerance to diseases and stress induced by environmental changes (Jean *et al*., 2004).

Another method to control infectious diseases is the use of vaccines and immunostimulants. Aquatic invertebrates and fish larvae cannot rely on an acquired immune system to combat disease, but have to rely on the innate immune system (Kurtz and Franz, 2003; Little and Kraaijeveld, 2004) consisting of cellular and humoral components (*e.g.* lysozymes, lysosomal enzymes, lectins, or antibacterial components) that interplay to recognise and eliminate foreign microorganisms and pathogens (Bachère, 2003). Vertebrates in later life stages and some invertebrates present both innate and acquired immunity (Little and Kraaijeveld, 2004). The latest immunity is adaptive,

flexible, specific and based on immunoglobulins and specified immune cells (in vertebrates) that improve the efficiency of the host's immune response during the second encounter with a pathogen (Moret and Siva-Jothy, 2003).

Vaccination can induce long-lasting protection through immunological memory and needs primary challenge with an antigen (Smith *et al.,* 2003). Application of vaccines in adult fish with fully developed immune system is very successful (Alabi *et al*., 2000; McLauchlan *et al.*, 2003; Irie *et al*., 2005). However, vaccines are not available for all pathogens, usually involve stressful handling of animals, and are ineffective with most invertebrates and early life stages of vertebrates (Olafsen, 2001).

Immunostimulants are naturally occurring compounds that modulate the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens (Bricknell and Dalmo, 2005).

The use of immunostimulants can improve the innate defense of animals. It does not require a specific response to a defined antigen and provides resistance to pathogens during periods of high stress, such as grading, reproduction, sea transfer and vaccination. The immunomodulation of larval fish has been proposed as a potential method for improving larval survival by increasing the innate responses of the developing animals until its adaptive immune response is sufficiently developed to mount an effective response to the pathogen. Several products, such as β-glucans, chitin, mannoproteins, peptidoglycans, alginate and bacterial components (*e.g.* lipopolysaccharides) are being applied in vertebrate and invertebrate cultures, to induce protection against a wide range of diseases (Boonyaratpalin *et al.,* 1995; Sung *et al.,* 1996; Itami *et al.,* 1998; Alabi *et al.,* 1999; Sritunyalucksana *et al.,* 1999; Takahashi *et al.,* 2000; Burgents *et al.,* 2004; Misra *et al.,* 2004; Skjermo and Bergh, 2004; Wang and Chen, 2005).

Saccharomyces cerevisiae, which has been found to be a good immune enhancer in some aquatic organisms (Siwicki *et al.,* 1994; Ortuño *et al.,* 2002; Li *et al.,* 2003, 2004), is an excellent source of β-glucans and chitin. The latter compounds are the major compounds in the yeast cell wall together

with mannoproteins (Magnelli *et al.,* 2002). Application of yeast cells as immunostimulants has many advantages. Firstly they can be produced rapidly, easily and inexpensively, and at the same time, they are very stable and can be obtained as by-products from other industries. Yeast also enjoys the GRAS status (GRAS: generally regarded as safe), so that no negative effects are expected neither on the animals nor on the environment. Moreover, there is no need to isolate its components, which consists mainly of cell wall sugars (β-glucans, mannoproteins and chitin), all well-proved immunostimulant extracts. The combination of all the yeast cell compounds, not only cell wall sugars but also vitamins and genetic material, might provide a mixture assuring an optimum physiological status in fish due to multiple interactions, particularly with regards to the immune system (Kulkarni *et al.,* 1987; Rudolph *et al.,* 1990; Cerra *et al.,* 1991). Moreover, yeasts are easily genetically manipulated enabling various modifications of the composition of the yeast cell-wall through the construction of gene deletion mutants. All these scientific and economic reasons make further investigation into baker's yeast *S.cerevisiae* and other industrially used microorganism a worthy topic of research since they may constitute an inexpensive alternative to the expensive isolated components usually used in fish diet formulations.

Although there are many studies published on the application of immunostimulants in aquaculture, they have mostly been performed under xenic conditions where it is very difficult to separate the effect of the feed compositions from the effect (or interaction) of the accompanying microbiota (*i.e.* nutritional or probiotic effects as reported by Marques *et al.,* 2005). In addition, the beneficial effects of immunostimulants in many cases have been questioned due to poor experimental design and absence of a reliable statistical analysis (Smith *et al.*, 2003). Therefore, Marques *et al*. (2004) have recently developed and standardized an *Artemia* gnotobiotic test system allowing to study the effect of food composition on survival and growth of *Artemia* in the presence or absence of a pathogen, providing a framework to acquire knowledge on host-microbial interactions.

III. *Artemia*

3.1. *Importance of Artemia to aquaculture*

Regardless of the vast improvement in fish nutrition industry there is still no artificial feed formulation available to completely substitute for *Artemia.* In fact, *Artemia* remains essential in most marine finfish and shellfish hatchery operations especially during the earliest life stages (Kolkovski *et al.*, 2004). *Artemia* is the most widely used live feed in larviculture due to its high nutritional quality (Sorgeloos *et al.*, 1986) and ease of use. Annually, more than 1,500 metric tonnes of dry *Artemia* cysts are marketed worldwide to feed fish and shellfish (Dhont and Sorgeloos, 2002). Shrimp hatcheries are the major consumers of *Artemia* cysts (80 to 85% of the total sales), mainly in China, South East Asia, Ecuador and other Latin-American countries.

3.2. Particular characteristics of Artemia

The brine shrimp *Artemia* is a small crustacean occurring almost worldwide in natural brine lakes or salt works. *Artemia* are extremely osmotolerant and is mostly found in salinities ranging between 45g/l and 200g/l, although they are able to live in brackish and supersaturated waters 340g/l) (Van Stappen, 1996, 2002). *Artemia* are also adapted to widely changeable temperature (6-35°C) and ionic composition, and their pH tolerance varies from neutral to highly alkaline (Van Stappen, 1996, 2002). They can produce cysts (a dormant embryo covered by a three-layered shell) through oviparous reproduction when the environmental conditions are harsh (Criel and MacRae, 2002). These cysts are easily collected in high quantities, since most of them float. After proper processing, they can be stored for several years at room temperature, remaining in diapause or staying dormant as long as they are kept dry and under anaerobic conditions (Van Stappen, 2002). Under optimal hatching conditions, the embryo becomes activated within the cyst shell and, within 24h, the shell breaks and nauplii (or Instar I) of 0.4-0.5mm hatch out of the cyst (Van Stappen, 1996). After about

8h, the Instar I nauplius moults into the 2nd larval stage (Instar II) and is now able to take up exogenous particles. The larva grows through 15 moults and becomes differentiated into male or female (at least for sexual species) after the tenth moult (Criel and MacRae, 2002). The maximum *Artemia* length is usually around 8-10mm for males and 10-12mm for females and their thickness approximates 4mm (including the legs) for both sexes (Criel and MacRae, 2002). Decapsulated cysts, nauplii and adults are commonly used to feed aquaculture species depending on the mouth size of larvae or fry. *Artemia* present particular feeding characteristics (continuous, nonselective and particle-filter feeder) that offer interesting opportunities for its use as live feed in aquaculture (Dobbeleir *et al.*, 1980). This animal can ingest small feed particles ranging from 1 to 50μm in size (Dobbeleir *et al.*, 1980) such as microalgae (Sorgeloos *et al*., 1986), baker's yeast, dried microalgae, organic detritus, bacteria and waste products from the food industry (*e.g.* rice bran, corn bran, soybean pellets, whey powder, etcetera) (Dobbeleir *et al.*, 1980; Sorgeloos *et al*., 1986). The peculiar feeding characteristic of the brine shrimp enables its use as a vector for delivering different substances to aquatic organisms using the bioencapsulation technique, such as nutrients, pigments (Sorgeloos *et al*., 2001), antimicrobial agents (Dixon *et al.*, 1995), vaccines (Campbell *et al.*, 1993), and probionts (Gatesoupe, 1994).

3.3. Advantages of using Artemia as test organism

Artemia has been suggested by many authors as a model organism for studying the biology of infections, host-microbes interactions or the effect of chemotherapy agents against diseases occurring in penaeid shrimp, lobsters and other crustaceans (Overton and Bland, 1981; Criado-Fornelio *et al*., 1989; Verschuere *et al*., 1999, 2000b, Marques *et al.,* 2004). *Artemia* was also used as a model to study regulation of gene expression during embryonic development (Escalante and Sastre, 1994). According to Marques *et al.* (2005), *Artemia* has several striking biological characteristics and advantages, enabling their potential use as model system for basic research in animal biology, such as: (i) the possibility to be cultured under axenic and gnotobiotic conditions

using various type of feed sources with a simple experimental apparatus (Verschuere *et al.*, 1999, 2000a,b); (ii) short generation time (2-3 weeks), although under optimal conditions the brine shrimp can live for several months, they can grow from nauplius to adult in as little as 8 days (Van Stappen, 1996); (iii) availability of large quantities of cysts, different species and strains from all continents (and hence different genetic background, Bossier *et al*., 2004); (iv) small-sized organism that can be easily cultured at high density and/or on a small scale, using very simple culture systems.

The success of culturing *Artemia* partly depends on the establishment of a favourable microbial environment. Several pathogens have already been reported in literature, *e.g.*: *Leucothrix mucor* (Solangi *et al*., 1979); *Vibrio alginolyticus* (Soto- Rodriguez *et al.*, 2003a; Villamil *et al*., 2003); *Vibrio parahaemolyticus* (Rico-Mora and Voltolina, 1995; Orozco-Medina *et al*., 2002); *Fusarium solani* (Criado-Fornelio *et al.*, 1989); *Vibrio proteolyticus* (Verschuere *et al*., 1999, 2000b); *Vibrio harveyi* or *Vibrio campbellii* (Roque and Gomez-Gil, 2003; Soto-Rodriguez *et al.*, 2003a,b); and *Vibrio vulnificus* (Soto-Rodriguez *et al.*, 2003a). Contrary to pathogenic or opportunistic bacteria, there are some favourable bacteria, which provide many advantages to the host by enhancing its survival rates, growth and the overall health condition. Indeed, bacteria may constitute a source of essential proteins, amino acids, vitamins and active enzymes (Intriago and Jones, 1993; Gorospe *et al*., 1996) and can provide probiotic effect as well (Verschuere *et al*., 2000a).

IV. Research objectives and thesis outline

Several solutions have been pursued to control diseases in aquaculture. However, results are often insufficiently conclusive because the influence of fluctuating culture conditions, especially the nature of the occurring microbial community, are not adequately controlled. Therefore, the validation of the efficacy of any new strategy requires standardized and controlled conditions.

The use of gnotobiotic systems is an excellent tool to study a variety of phenomena (Marques *et al.,* 2006). With such systems it is possible to extend the understanding of the mechanisms involved in

host-microbe interactions, evaluate new treatments and design effective and reproducible experimental conditions aimed at controlling diseases in aquaculture practices. Eventually, the findings can be verified in non-gnotobiotic conditions.

The general objective of the present study is to investigate the protective nature with respect to pathogenic bacteria of isogenic yeast mutants in *Artemia.* Since various yeast mutants provide different cell wall compositions (β-glucans, mannoproteins and chitin), a selection of yeast mutant strains was used as protective agents in a gnotobiotic *Artemia* test system controlling the pathogenicity of *Vibrio* present in the *Artemia* culture.

In **Chapter I (Introduction)** an overview is presented on the general aspects of some alternative and/or new methods that are being considered for use in aquaculture to control diseases. In addition, some specific characteristics of *Artemia* are described to elucidate the strong potential of this organism as a model organism in aquaculture research.

Since β-glucans are the most widely-used immunostimulants and immunostimulatory effects of yeast cells are mainly attributed to the presence of these compounds in the cell wall, in **Chapter II (β-glucans as immunostimulant in vertebrates and invertebrates)** a special overview is made about these products. This chapter summarizes information on the structure and sources of β-glucans, their mode of action as well as their application in vertebrates and invertebrates.

In **Chapter III (Influence of different yeast cell-wall mutants on performance and protection against pathogenic bacteria (*Vibrio campbellii*) in gnotobiotically-grown *Artemia)*** the nutritional and/ or protective properties of yeast cells with respect to *Artemia* (including a *Vibrio* challenge test) are verified. Therefore, a series of axenic yeast mutant strains with different cell wall composition, harvested in the exponential or stationary growth phases are tested.

A complementary study to the work accomplished in the previous chapter is presented in **Chapter IV** (**Anti-infectious potential of beta-mercaptoethanol treated baker's yeast in gnotobiotic *Artemia* challenge test).** Since the external mannoprotein layer of the yeast cell wall is supposed to be the main barrier to yeast digestion by *Artemia*, a chemical treatment using 2-

mercapto-ethanol (2ME) is applied to a series of yeast mutant strains (the same selection as used in chapter III) in order to break disulfide linkages connecting mannoprotein molecules in an attempt to improve yeast digestibility resulting in an increased accessibility of β-glucans to *Artemia.* Chemically-treated yeast cells are fed to *Artemia* and animal performance is evaluated and compared with untreated yeast-fed *Artemia* in a gnotobiotic challenge test system.

Instigated by studies that reports a beneficial effects of immunostimulants when applied either before, simultaneous or even after challenge with a pathogen, **Chapter V (The protective effect against *Vibrio campbellii* in *Artemia* nauplii by pure β-glucan and isogenic yeast cells differing in β-glucan and chitin content operated with a source-dependent time lag)** reports on the verification of the protective nature of two products (mnn9 yeast cells and/ or pure β-glucan) when they are applied to *Artemia* at different time intervals before or after challenge.

Making use of the advantages of the gnotobiotic *Artemia* test system, a collection of putative commercial immunostimulants is tested in **Chapter VI (Enhanced disease resistance in *Artemia* by application of commercial β-glucans sources and chitin in a gnotobiotic *Artemia* challenge test)**.

In **Chapter VII (General Discussion and Future Research)** the main results obtained in the previous chapters and conclusions drawn throughout the thesis are discussed in the framework of the research objectives. Additionally, the perspectives for further research are outlined.

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CHAPTER II

β-glucans as immunostimulant in vertebrates and invertebrates

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I. Introduction

A variety of polysaccharides from a variety of sources have the ability to stimulate the immune system, so behaving as immunomodulators. Interest in glucans has increased after experiments showing that zymosan stimulates macrophages via the activation of the complement system (Fitzpatrick and DiCarlo, 1964). Pharmacologically they are classified as biological response modifiers (BRM). Different physicochemical parameters, such as solubility, primary structure, molecular weight, branching and polymer charge influence the biological activities of β-1,3-glucans (Bohn and BeMiller, 1995). The immunomodulating effects of β-glucans are well established during the development of immune reactions (Vetvicka and Sima, 2004). Original studies on the effects of β-1,3-glucans on the immune system focused on mice (Suzuki *et al*., 1990; Kournikakis *et al.*, 2003). Subsequent studies demonstrated that β-1,3-glucans have strong immunostimulating activity in a wide variety of other species, including earthworms (Beschin *et al*., 1998), shrimps (Duvic and Söderhäll, 1990), fish (Anderson, 1992), rats (Feletti *et al*., 1992), rabbits (Kennedy *et al.*, 1995), guinea pigs (Ferencik *et al*., 1986; Drandarska *et al.*, 2005), sheep (Waller and Colditz, 1999), pigs (Dritz *et al.*, 1995; Li *et al.*, 1996; Hiss *et al.*, 2003), cattle (Buddle *et al.,* 1988) and humans (Kougias *et al.*, 2001). Based on these results it has been concluded that β-1,3-glucans represent a type of immunostimulant that is active across the evolutionary spectrum, likely representing an evolutionarily conserved innate immune response directed against fungal pathogens. Invertebrates have evolved a wide variety of active defense mechanisms enabling them to use their highly effective innate defense pathways to protect themselves against invading pathogens despite the absence of an adaptive immune system based on lymphocytes or antibodies (Vetvika *et al.,* 2004). Fungal β-glucans can induce all of the major anti-microbial immune mechanisms found in invertebrates, including the humoral, cellular and phenoloxidase responses (Brown and Gordon, 2005). The majority of these responses rely on protease cascades which are

initiated by Pahogen Associated Molecular Pattern (PAMP) recognition in the haemolymph (Brown and Gordon, 2005).

In vertebrates, the immunomodulating abilities of β-glucans are thought to stem from their ability to activate leukocytes, but there is some confusion about their precise biological effects (Brown and Gordon, 2003). This has occurred through the use of different β-glucans which vary in their origin, molecular structure and purity, parameters which have been shown to influence their activity (Bohn and BeMiller, 1995). A number of cellular receptors have been implicated in these activities, including Dectin-1, CR3, lactosylceramide, scavenger receptors and Toll-like receptors 2 and 6 (TLR) (Brown and Gordon, 2003). When the receptor is engaged by β-glucans, the cells become more active in engolfing, killing and digestion of bacteria and at the same time they secrete signal molecules (cytokines), which stimulate the attraction and the formation of new white blood cells as well as the activation of these cells (Gantner *et al.,* 2003). Although the primary role of β-glucan recognition appears to be the initiation of immune responses for the control of fungal pathogens, the receptors and mechanisms by which this is achieved differe significantly between vertebrates and invertebrates. In vertebrates, the recognition and response to these structures are initiated by cell surface receptors, whereas this process occurs primarily in the haemolymph in invertebrates (Brown and Gordon, 2005).

2. Structure and sources

β-1,3-glucans are structurally complex homopolymers of glucose, usually isolated from cell walls of bacteria, mushrooms, algae, cereal grains, yeasts and fungi (Zekovic and Kwiatowski, 2005). The number of individual β-glucans is almost as great as the number of sources used for isolation (Vetvicka and Sima, 2004). Their activity is influenced by their degree of branching, size and their molecular ultrastructure. The most active ones have a common structure: a main chain consisting of (l-3)-linked β-D-glucopyranosyl units along which are randomly dispersed single β-D-glucopyranosyl units attached by l-6 or 1-4 linkages (Bohn and BeMiller, 1995; Zekovic and

Kwiatowski, 2005). Not only the type and frequency of these branches vary depending on the different sources of the β-glucans, but also the length of their main chain (Bohn and BeMiller, 1995) and their activities (Wagner *et al.,* 1988; Jamas *et al.,* 1991; Kraus & Franz, 1992). Due to this variation β-1,3-glucans occur with variable molecular weight (MW) and degree of branching (DB) (Williams, 1997). As a result, β-1,3-glucans have specific molecular ultrastructures *i.e.* triple helix, single helix or random coil structures (Ohno *et al.,* 1988a*).* Data suggest that higher ordered structures like triple helices are responsible for the immunomodulating activity (Hamuro *et al.,* 1971; Norisuye, 1985; Ohno *et al.,* 1987a; Kojima *et al.,* 1986; Maeda *et al.,* 1988). Furthermore β-glucans with a degree of branching of 0,2 – 0,33 seem to be the most active ones (Bohn and BeMiller, 1995) (see Table 2.1). In addition, evidences suggest that the activity of the polysaccharides is also dependent on their size, with high molecular weight (100-200 kDa) fractions being most active, while fractions from the same source with molecular weights of 5-10 kDa show no activity (Blaschek *et al.,* 1992; Fabre *et al.,* 1984; Kojima *et al.,* 1986). Yet, a polysaccharide from a *Pythium aphaniderma* glucan, with a molecular weight between 10-20 kDa has been reported to exhibit antitumor activity (Bell *et al.,* 1992; Gomma *et al.,* 1992; Kraus *et al.,* 1992). A conclusion for these conflictory data was made by Zhang and coworkers (2005). They demonstrated hat samples with a lower MW had higher activity *in vitro*, while higher MW samples were more active *in vivo*. Other data however indicate that it is the distribution of the glucosyl units along the backbone chain that confers immunomodulating activity (Misaki *et al.,* 1993; Williams, 1997). A heptasaccharide is the smallest unit ligand recognized by macrophage glucan receptors (Lowe *et al.,* 2001). Depending on the structure of the β-glucans, they can be divided in three main groups with different properties: soluble, gel-forming and particle-forming. In β-1,3-glucans the length of unbranched chains of glucose residues has a marked effect on solubility properties. Soluble laminarin has a low degree of branching and an average chain length ranging from 7 to 10 glucose residues (Fleming *et al.*, 1966). By contrast, the molecules of insoluble laminarin are essentially linear and contain about 15-20 residues. The β-1,3-glucans with a high degree of polymerization

(DP> 100) are completely insoluble in water (Zeković *et al.,* 2005). The solubility increases as the degree of polymerization of β-1,3-glucan is lowered. In fact, the solubility of β-1,3-glucans appears to depend both on degree of polymerization and on the length of side substituted branches. The frequency of side branches determines the solubility of different β-1,3-glucans (Fleet *et al.,* 1976). For laminarin, however, even a single (1→6)-β linked glucose side chain can transform the glucan into a more soluble form compared to its unbranched molecule (Nelson and Lewis, 1974). The most studied commercial immunomodulating β-1,3-glucans are summarized in Table 1.

Table 2.1 Commercially used β-1,3-glucans and their characteristics

Origin	β-glucan	Chemical structure	Degree of substituton or branching frequency	Degree of polymerization	MW (kDa)	Solubility	References
FUNGI: *Coriolus versicolor (Turkey tail)* *Lentinus edodus (Shiitake)*	Krestin (Kureha, PSK)	β-1,4-D-glucan-protein-complex with β-1,6-glucopyranosyl side chains	1/5		100	Soluble? (triple helix)	Ooi and Liu, 2000 Ohmura *et al.*, 2006
Sclerotium glucanicum *Sclerotium sclerotiorum*	Lentinan	β-1,3-D-glucan with β1,6-glucopyranosyl side chains	2/5		400-800	Gel (triple helix)	Chihara *et al.*, 1969 Ooi and Fang, 2000
Schizophyllum commune	- Scleroglucan - SSG	β-1,3-D-glucan with β-1,6-glucopyranosyl side chains	1/3 Highly branched		- 1600-5000 - 200-2000	Soluble (triple helix)	Palleschi *et al.*, 2005 Rice *et al.*, 2005
Grifola frondosa (Maitake) *Poria cocos* Wolf	Schizophyllan (SPG, sonifilan, sizofiran, sizofilan)	β-1,3-D-glucan with β1,6-glucopyranosyl side chains	1/3	230	306-450	Gel (triple helix)	Miura *et al.*, 1995 Bot *et al.*, 2001 Kubala *et al.*, 2003
Glomerella cingulata	Grifolan (GRN, grifolan LE)	β-1,3-D-glucan with β-1,6-glucopyranosyl side chains	1/3		500	(triple helix + single)	Adachi *et al.*, 1989 Adachi *et al.*, 1994 Ishibashi *et al.*, 2001
Monilinia fructigena	Pachyman	β-1,3-linked glucan with a small amount of β-1,6-linked branching glucan	1,0-1,3	255-	50-270 80-120 189	Insoluble	Hattori *et al.*, 1992 Ding *et al.*, 1999 Osmond *et al.*, 2001 Yiannikouris *et al.*, 2004 Wang *et al.*, 2004

YEAST: *Saccharomyces cerevisiae*	Betafectin (PGG)	PGG (poly-β-1,6-glucotriosyl-β-1,3-glucopyranose-glucan) (triple helix)	0,5		150-?	Soluble (non-uniform?)	Ondetonk *et al.*, 1992 Bohn and Bohlfiller, 1995 Kernodle *et al.*, 1998 Patchen *et al.*, 1998 Dellinger *et al.*, 1999 Walsknill *et al.*, 1999
Candida albicans	MacroGard	β-1,3-D-glucan with β-1,6-glucopyranosyl side chains			200	Soluble (sMG) and particle (pMG) (non-uniform)	Hetland and Sandven, 2002 Instanes *et al.*, 2004
	Zymosan	β-glucan (β-1,3-linked and β-1,6-linked glucan moieties) and mannan, protein, and melenic acid	0,03-0,2		<200?	Particle (non-uniform?)	Manners *et al.*, 1973 Williams *et al.*, 1986 Young *et al.*, 2003 Frassnelli *et al.*, 2005
	Glucan-phosphate	Glucan-phosphate (β-1,3-glucan-P)	0,10-0,14		157 92	Soluble (single helix)	Miller *et al.*, 1996 Sherwood *et al.*, 2001 Rice *et al.*, 2005

3. Biochemical background of β-glucan action

The immunomodulatory activities of β-glucans are still far from being understood, particularly those of the intermediate MW glucans, but recent studies have started to shed some light on the mechanisms behind the proinflammatory response induced by large MW and particulate β-glucans. β-glucans, along with mannans and other cell wall components play an important role in recognition of fungal pathogens. Indeed, in vertebrates many of the β-glucan receptors, including CR3, lactosylceramide and Dectin-1, have been shown to contribute to the recognition and phagocytosis of these organisms (Brown and Gordon, 2003). The action of β-glucans requires target cells, mainly myeloid cells, which have an important role in innate as well as adaptive immunity. The effects on macrophages for example exist of better phagocytosis, the production of reactive oxygen species (ROS), secretion of cytokines and a higher processing and presenting of antigens (Czop, 1986; Meira *et al.*, 1996). The direct activation of other immune cells, such as NK cells and lymphocytes, can be considered as secondary (Leung *et al*., 2006). β-glucans are considered as strong mitogens inducing proliferation of peripheral blood mononuclear cells (PBMC). Furthermore they activate NK cells, induce T-cell mediated cytotoxicity, release of cytokines such as interferons and interleukins and phagocytosis of neutrophils (Bohn and BeMiller, 1995). β-glucans also heighten the non-immunological resistance of the host through the stimulation of acute phase proteins production.

What probably contributes to the biological activity of β-glucans is their long presence in vertebrates due to the absence of specific β-glucanases (Miura *et al.*, 2003). As a consequence β-glucans are available in the cells for weeks or even months and only get degraded via slow oxidative processes.

Hence, the action of β-glucans depends also on the dose that is administered and the time point of this administration. Too low doses have no effect and too high doses have a suppressive effect. For every animal, the optimal dose has to be determined (Vancaeneghem *et al.*, 2000).

3.1. β-glucan binding proteins

The innate ability to detect pathogens is essential for multicellular existence, and has been achieved through the evolution of germ-line encoded receptors which can recognize non-self structures, the so-called 'pattern recognition receptors' (PRRs) (Janeway, 1992). These receptors evolved to recognize conserved products of microbial metabolism produced by microbial pathogens, but not by the host. Recognition of these molecular structures allows the immune system to distinguish infectious non-self from non-infectious self. The structures recognized by these receptors, termed the pathogen-associated molecular patterns (PAMPs), are not found in the metazoa and are thought to be normally essential for the survival of the microbial pathogen. The best-known examples of PAMPs are bacterial lipopolysaccharide, peptidoglycan, lipoteichoic acids, mannans, bacterial DNA, double-stranded RNA and β-glucan of fungi (Medzhitov and Janeway, 2000). Recognition of these structures triggers responses designed to protect the host from the invading pathogen, forming part of the innate immune system found in all higher organisms (Brown and Gordon, 2003). Surprisingly, the recognition of β-glucans by vertebrates differs significantly from that of invertebrates. Vertebrate recognition of soluble and insoluble β-glucans appears to occur exclusively via a number of cell surface receptors and although complement opsonization does contribute to the recognition of particulate glucans, no plasma molecules recognizing this carbohydrate structure have been identified (Brown and Gordon, 2005). In contrast, the recognition of β-glucans appears to occur primarily in the haemolymph of invertebrates via a number of completely unrelated proteins, such as horseshoe crab factor G and gram-negative binding proteins (GNBPs). Only one cellular receptor has been shown to recognize β-glucans in invertebrates, the *Drosophila* scavenger receptor (dSRCI). Despite these differences, the recognition of β-glucans in both systems results in the triggering of immune responses, designed primarily for the control of fungal pathogens.

3.2. Vertebrate cell surface receptors for β-glucans

The evidence for cellular β-glucans receptors originated from the recognition of non-opsonized zymosan by human monocytes on a β-glucans dependent way (Brown and Gordon, 2003). Since then the β-glucan receptor activity has been identified on immune and non-immune cells, like monocytes, macrophages, neutrophils, Langerhans cells, eosinophils, NK cells, endothelial cells (Lowe *et al.*, 2002), epithelial cells (Ahren *et al.*, 2001) and fibroblasts (Kougias *et al.*, 2002) (Brown and Gordon, 2005). The non-opsonized recognition of β-glucans by these cells is attributed to complement receptor 3 (CR3), lactosylceramide receptor, scavenger receptor and dectin-1 receptor (Ross *et al.*, 1987, Rice *et al.*, 2002, Brown *et al.*, 2003; Gantner *et al.*, 2003) (see Fig. 2.1).

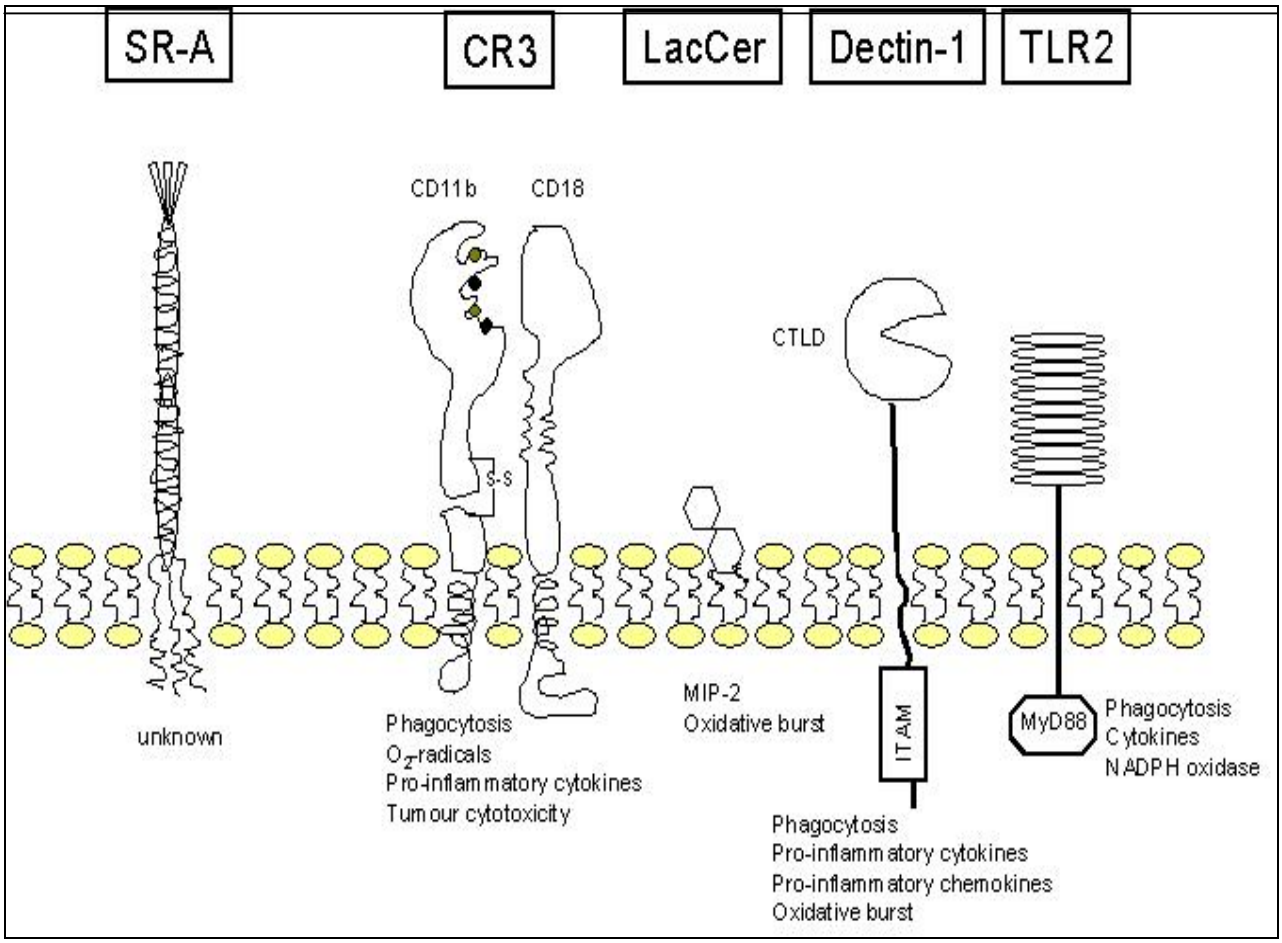

Fig. 2.1 Overview of the vertebrate β-glucans cell surface receptors. The structures of the scavenger receptor type A (SR-A), the complement receptor 3 (CR3), Lactosylceramide, Dectin-1 and Toll-like receptor 2 (TLR2) are shown and also the effects through activation of these receptors by β-glucans (Modified from Brown and Gordon, 2005).

Cellular recognition is mediated by a combination of these receptors, although only dectin-1 has been described to play a role in a broad outline of the biological response, to these carbohydrates. Some leukocyte populations express different receptors in the recognition of β-glucans. The mannose binding lectines and also TLR2 and TLR6 have also been described to play a role in the recognition of β-glucans (see Fig. 2.1). These TLRs do require more factors to start a response. The first receptor mentioned in literature for binding β-glucans was the Membrane associated component 1 (Mac-1), also called complement receptor 3 (CR3). This leukocyte-receptor belongs to the β_2-integrin family and consists of two chains, an α_m-chain (CD11b) and a β_2-chain (CD18a) (Ross *et al.*, 1987; Thornton *et al*., 1996; Xia *et al.*, 1999). The alfa-chain has two binding sites. The first binding site, the I-domain, binds the inactive form of C3b (iC3b), the intercellular adhesion molecule (ICAM-1), some extracellular matrix proteins and fibrinogen (Diamond *et al.*, 1993). The second binding site, a cation-independent lectin site on CD11b, binds β-glucans (Thornton *et al*., 1996; Xia and Ross, 1999). The part of the β-glucans recognised by CR3 is different from species to species. For example, in Atlantic salmon, a fragment of 3 consecutive β-1,3-glucose molecules is recognised and no β-1,6-linkages (Engstad and Robertson, 1994). In contrast, on human monocytes a fragment of seven beta-1,3-glucose molecules is bound (Lowe *et al.*, 2001). CR3 is present on the surface of macrophages, NK cells, microglia cells and some lymphocytes (Vancaeneghem *et al.,* 2000; Brown and Gordon, 2003) . The expression of CR3 is influenced by IFN-γ, IL-1, vitamin D_3 and PGE_2. Especially IFN-γ stimulates the expression (Konopski *et al.*, 1993). Moreover it also stimulates the uptake of the bound β-glucans, with a negative feedback in case of abundance, and so activates the production of TNF-α. IL-1 and vitamin D_3 also stimulate the expression of CR3, while PGE_2 does the opposite (Konopski *et al*. 1993; Poutsiaka *et al*, 1993). Leucocytes that don't have CR3 on their surface, still react in a same way to β-glucans, so CR3 is probably not the most important β-glucans receptor.

A second receptor for β-glucans mentioned in literature, is lactosylceramide (LacCer) or CDw17, a glycosphingolipid, occurring in the plasma membrane of polymorphonuclear leucocytes and

macrophages. Its structure consists of a hydrophobic ceramide lipid and a hydrophilic sugar, forming micro domains in the plasma membrane. Monoclonal antibodies to this lactosylceramide inhibited the binding of radioactive labelled β-glucans to macrophages (Hahn *et al*., 2003). Also mentioned in the literature as a potential receptor for β-glucans would be a particular, not yet identified scavenger receptor. The interaction of monocyte membranes with scavenger-ligands can be inhibited by adding soluble β-glucans (Rice *et al*., 2002). Scavenger receptors include a heterogeneous group of molecules which have the possibility to recognise and respond to modified lipoproteins of low density, polyanionic ligands, lipoteichoic acid and pathogens (Rice *et al*., 2002). These receptors are expressed on the surface of macrophages, dendritic cells, endothelial cells and smooth muscle cells. But the major β-glucans receptor now considered is Dectin-1 (Brown *et al.*, 2002). This receptor has been discovered by screening the cDNA of a mouse macrophage cell line with the β-glucans rich particle zymosan (Brown and Gordon, 2001). A single receptor was isolated and the DNA sequence was determined. They found a dendritic cell (DC)-associated C-type lectin, also called Dectin-1, belonging to the C-type lectin (CTL) family. CTL receptors belong to the bigger family of PRR, recognise conserved PAMP's and help to make a distinction between self and non-self modulated immune responses. CTL are expressed on the surface of a broad spectrum of cells, like dendritic cells and other antigen-presenting cells (APC). Many CTL receptors contain signalling motifs in their cytoplasmatic domain like an ITAM or ITIM motif, indicative of their involvement in activation of cell signaling. Dectin-1 is a type II transmembrane glycoprotein with an extracellular C-type lectin-like domain at the COOH-end, a stalk, a transmembrane domain and a short cytoplasmatic tail with an ITAM-motif at the NH2-end (Herre *et al*., 2004). Although dectin-1 belongs structurally to the CTL family with one carbohydrate recognition domain (CRD)-motif at the COOH-end, it does not function as a conventional C-type lectin by his ligand specificity. This receptor has been described in mouse, man and cattle and is expressed on the surface of various cells, including dendritic cells, macrophages, monocytes, neutrophils, NK cells and a subset of T-cells (Brown and Gordon, 2001). Dectin-1 is alternatively spliced so different isoforms exist of this

receptor, two major isoforms in man, mice and cattle (Willment *et al*., 2001; Heinsbroek *et al.*, 2006; Willcocks *et al., 2006*) and 6 minor isoforms in human (Willment *et al*., 2001). The major isoforms A and B differ in their neck region, which is absent in isoform B. Mouse Dectin-1 is located on chromosome 6, expressed by the CLEC7A gene. His size is 28kDa or 244 amino acids or 735 base pairs (Arizumii *et al*., 2000; Willment *et al*., 2001; Herre *et al*., 2004; Heinsbroek *et al.*, 2006). The expression of mouse Dectin-1 can be up-regulated by GM-CSF and IL-4 and down regulated by IL-10 and LPS (Heinsbroek *et al.*, 2006). Human Dectin-1 (hDectin-1) is structurally analogous to mouse Dectin-1, but in contrast more isoforms are formed by alternative splicing. The deletions evolved through this alternative splicing are known and lead to a truncated protein, except for hDectin-1E (Willment *et al.*, 2001). Splicing seems to be regulated in different cell types, so the same isoforms don't occur in every cell type. HDectin-1A has 247 AA (744 bp) and hDectin-1B 201 AA (606 bp). The gene CLEC7A is located on chromosome 12 and consists of 6 ORF's with 6 exons and 5 introns. Hdectin-1 is also expressed on B-cells and eosinophils in contrast with mouse Dectin-1 (Willment *et al.*, 2005). Very recently also the bovine dectin-1 has been fully characterised (Willcocks *et al*, 2006). One demonstrated the existence of two major isoforms A and B where the smallest one also lacks the neck region by analogy with man and mouse. Bovine Dectin-1 is located on chromosome 5 and encloses 247 AA/ 744 bp (isoform A) or 201 AA/ 606 bp (isoform B). In contrast with hDectin-1 bovine T and B lymphocytes seemed to be negative for Dectin-1 and no independent regulation of the two isoforms was seen in different cell types (Willcocks *et al*, 2006).

Lately researchers have also discovered a role for TLR2 and 6 in β-glucans recognition through dectin-1. TLRs include at least 13 membrane signalling molecules, belonging to the PRR. They recognize strong conserved sequences on pathogens, so called PAMPs, where after inflammatory signals occur (Trinchieri and Sher, 2007). They are integral membrane proteins located on the cell surface or in intracellular compartments. They all contain an N-terminal LRR, responsible for ligand binding, and a C-terminal TIR domain, necessary for the initiation of intracellular signalisation. TLR can form homo- or heterodimers with other TLR or other receptors. As a

consequence, their repertoire of ligands increases enormously (Eddie *et al*, 2005). Evidence suggests that, beside Dectin-1, also TLR2 and TLR6 play an important role in the response on zymosan. TLR2 reacts directly on this particle, but forms a functional pair with TLR6 to induce the production of cytokines and chemokines through MyD88 and NF-κB. However it seems that the β-glucans receptor Dectin-1 is necessary for the recognition and inflammatory response on these carbohydrates. In addition, these receptors strengthen each other's response. Dectin-1 and TLR2/6 collaborate and work synergistically (Gantner *et al*., 2003) (Mukhopadhyay *et al*., 2004).

3.3. Invertebrate recognition proteins (receptors)

A number of recognition proteins have been reported in invertebrates such as GNBPs/ βGRP, Hd-PGRP, Factor G and SR-CI.

The Gram-negative binding proteins (GNBPs)/βGRP (β-glucans receptor proteins) are perhaps one of the best characterized families of PRRs in invertebrates. These PRRs contain a C-terminal domain similar to bacterial β-glucanases, but lack enzymatic activity because of a number of amino acid substitution in the active site (Royet, 2004). In most insects, β-glucan recognition is mediated by an N-terminal extension of about 100 amino acids, which has also been shown to have immunomodulating activity (Fabrick *et al.*, 2004). Most of these proteins are secreted into the haemolymph, but at least one may be membrane bound via a GPI-linked anchor (Kim *et al.*, 2000). Expression of some of these proteins can be induced upon infection with yeast or bacteria (Jiang *et al.*, 2004). Although these proteins have been implicated in a variety of immune responses in invertebrates, including the activation of the prophenoloxidase (proPO) cascade and anti-microbial peptide production (Ferrandon *et al.*, 2004; Royet, 2004), the mechanism by which binding of β-glucan triggers these responses is unknown. The members of PGRPs (Peptidoglycan receptor proteins) family are primarily involved in the recognition of peptidoglycan, but two plasma PGRPs

from *Holotrichia diomphalia* (Hd-PGRP-1 and Hd-PGRP-2) have been shown to be capable of recognizing β-glucan as well. Hd-PGRP-1 is able to induce the prophenoloxidase cascade in the presence of β-glucan, suggesting that it may play a direct role in anti-fungal responses (Lee *et al.*, 2004). Horseshoe crab Factor G is a non-covalently linked heterodimer found in the haemolymph which responds specifically to β-glucan. The α subunit, which contains domains similar to bacterial glucanases and carbohydrate binding proteins, mediates the recognition of β-glucans, while the β subunit is a protease zymogen (Takaki *et al.*, 2002). In response to β-glucan, Factor G becomes activated by undergoing autocatalytic proteolysis and initiates activation of the proclotting enzyme and the coagulation cascade, leading to haemolymph clot formation (Muta *et al.*, 1995). SR-CI is a class C scavenger receptor that was identified and cloned from *Drosophila* haemocytes (Pearson *et al.*, 1995). This receptor was shown to recognize typical scavenger receptor ligands, described above, as well as intact Gram-positive and Gram-negative bacteria. Although able to recognize the soluble β-glucan, laminarin, the functional significance of this interaction is unclear as the receptor does not recognize intact fungal particles (Pearson *et al.*, 1995; Ramet *et al.*, 2001).

Proteins binding to the β-glucan have been identified in numerous arthropod species (Vetvicka and Sima, 2004). Their activity is usually to stimulate the PPO activation cascade. Subsequent purification and identification revealed similar properties: proteins containing carboxyl-terminal glucanase-like domain without enzymatic activity. There are suggestions that these proteins might develop from a primitive glucanase and later evolve into glucan-binding molecules without any enzymatic activity. GBPs (glucan binding proteins), sometimes also named as glucan-receptor, was also isolated from plasma of a silkworm, *Bombyx mori* (Yoshida *et al*., 1986). Later studies showed that these molecules are 30 kDa lipoproteins (Ujita *et al*., 2002). A different, high-density glucan-binding lipoprotein has been found in the white shrimp *Penaeus vannamei,* having only significant similarity to the GBP from the crayfish (Romo-Figueroa *et al*., 2004). Söderhäll's group isolated and characterized a GBP from the crayfish *Pacifastatus leniusculus* and found that this 40 kDa

protein has a strong similarity to bacterial glucanases and to the GBPs from *Eisenia foetida.* This protein bound both linear and branched glucans as well (Lee *et al*., 2000). A detailed study evaluated cDNA cloning, purification, properties and functions of a GBP from a moth, *Plodia interpunctella.* Functional data revealed that this GBP bound only to the 1,6-branched glucans (as it bound to laminarin, but not to curdlan). Hence, this GBP has two binding domains separated by a putative linker region, one for glucan and the second for the stimulation of the PPO cascade (Fabrick *et al*., 2004). GBPs are commonly found in crustaceans. They usually have a size of approximately 100 kDa and besides binding glucan, bacteria and haemocytes; they have a strong ability to act as opsonins (Cerenius *et al*., 1994).

The recently identified receptor of the toll family appear to have a major role in the induction of immune and inflammatory responses (Medzhitov *et al*., 2000). The first receptor of the toll family was identified in *Drosophila* as a component of a signaling pathway. In *Drosophila* activation of toll receptor by microbial infection triggers the rapid up-regulation of a variety of peptides with antimicrobial activity (Hoffmann *et al.,* 1999). Homologues of *Drosophila* toll have been identified in mammals and are referred to as toll-like receptors (TLRs) (Medzhitov *et al.* 1997; Rock *et al.,* 1998). The presence of TLRs in fish has also been shown (Stafford *et al.,* 2003). These finding suggested that TLRs may function as receptors of the innate immune system (Medzhitov *et al.*, 1997).

4. Immune activation pathways

Innate immunity plays a very important role in combating microbial infection in all animals. The innate immune response is activated by receptors that recognize surface determinants conserved among microbes but absent in the host, such as lipopolysaccharides, peptidoglycans and mannans (Medzhitov and Janeway, 1997). Upon recognition, these receptors activate multiple and complex signalling cascades that ultimately regulate the transcription of target genes encoding effector molecules. Importantly, different pathogens elicit specific transcription programmes that can now

be investigated by using microarray technology (De Gregorio *et al*., 2001; Huang *et al*., 2001; Irving *et al*., 2001).

4.1. Immune activation pathway in vertebrates

Although the mechanism of action of β-glucans in vertebrates has not been completely elucidated yet, it is known that binding of β-glucans to a pattern recognition receptor is crucial for its activity. β-glucans receptors described in vertebrates are the CR3, lactosylceramide, scavenger receptor, Dectin-1 and TLR2.

CR3 functions as a phagocytic receptor for a broad range of opsonized and non-opsonized pathogens, including iC3b-opsonised particulate β-glucans. When the β-glucans binds to the lectin domain of CR3, a conformational change develops via a tyrosine kinase- and magnesium-dependent mechanism, thereby exposing an epitope and so activating ('priming') the receptor (Ross *et al*., 1999). This activation leads to phagocytosis and the production of O_2-radicals and cytokines. Although the β-glucans binding site of CR3 has a broad range of sugar specificity, only the interaction with β-glucans will lead to activation (Xia and Ross, 1999). Since soluble and particulate β-glucans show a different activity, one suggests that these glucans might activate different signalling pathways. Soluble β-glucans would activate the same pathway as LPS, while particulate β-glucans would activate a different, still unknown pathway (Vancaeneghem *et al*., 2000).

The group of Hahn *et al.* (2006) suggests that binding of β-glucans to another receptor, lactosylceramide can induce the macrophage inflammatory protein (MIP)-2 in rat alveolar epithelial cells. Moreover, activation of NF-κB through this receptor would lead to an enhancement of the oxidative burst of human neutrophils (Zimmerman *et al*., 1998). Although the exact mechanisms of lactosylceramide are not known yet, a possible role of Lyn kinase has been mentioned.

A third group of receptors, the scavenger receptors (SRs) are involved in the homeostasis as well as immunity. Although a β-glucans specific scavenger receptor hasn't been identified, a few studies have demonstrated that soluble β-glucans can act as ligands for class A SRs. Vereschagin and coworkers (1998) demonstrated a protection against endotoxic shock when glucans bound to SRs on macrophages. The interaction between β-glucans and SRs is complex and is influenced by charge (Rice *et al.*, 2002).

The fourth and major β-glucans receptor Dectin-1 recognises soluble and particulate β-1,3/1,6-glucans, intact fungi and yeasts, and an undefined ligand on T-lymphocytes. Two amino acids, Trp^{221} and His^{223} in the carbohydrate recognition domain (CRD) are determined as necessary for the β-glucans interaction, although the exact mechanism of carbohydrate recognition by the non-classical C-type lectin-like domain is still unclear (Adachi *et al.*, 2004). Dectin-1 plays a significant role in the non-opsonized recognition, in contrast with MR and CR3, and has thus a central role in the innate recognition of these carbohydrate polymers and could be a potential therapeutically target for the development of new medicines (Brown and Gordon, 2005). Immediately after binding his ligand, Dectin-1 will phagocytose and endocytose this ligand and a signal cascade starts in the cytoplasm through the cytoplasmatic ITAM-motif but independently from the syk kinase (Brown and Gordon, 2005). Nevertheless, the ITAM-motif of Dectin-1 is not perfect. The consensus motif does contain two repeats of YXXL/I, but the distal repeat lacks the L/I. Still, tyrosine phosphorylation of this motif is necessary. This cascade results in the production of cytokines and chemokines such as TNF-α, CXCL-2, IL-2, IL_10 and IL_12 and reactive oxygen species. Herre and coworkers (2004) reported that syk kinase isn't strictly necessary for phagocytosis via Dectin-1, in spite of the presence of an ITAM-like motif. However, this syk kinase is required for the antimicrobial oxidative reaction through Dectin-1 (Underhill *et al*., 2005) and partially for cytokine production. The caspase-recruitment domain CARD9 plays a role in the linking activation of Dectin-1-syk to activation of NF-κB and in cytokine production in BM-DCs (Gross *et al.*, 2006). Underhill and coworkers (2005) found that there is only a fraction of cells establishing this syk

signalisation. This fraction can be manipulated through cytokines which play a role in the maturation of the immune response. Dectin-1 also recognises an unidentified ligand on CD4+ and CD8+ T lymphocytes and is able to stimulate their proliferation (Willcocks *et al*., 2006). The production of TNF-α through Dectin-1B is significantly higher than through isoform A. It seems that isoforms can influence the cytokine production (Heinsbroeck *et al.*, 2006). The mechanism is not known. Maybe the lack of a neck region establishes different interactions with other molecules? No knowledge exists of the function of the minor isoforms of hDectin-1 (Xie *et al.*, 2006), except for isoform E. Very recently, the function of this minor isoform was discovered. HDectin-1E contains a complete C-type lectin-like domain and an ITAM-like sequence, but lacks a part of the putative cytoplasmatic domain, the transmembrane region and the stalk. It is not expressed on the cell surface, because it lacks the transmembrane region. Instead it is soluble and remains in the cytoplasm. A Ran-binding protein (RanBPM) has been identified to colocalize with this isoform in the cytoplasm. This RanBPM can act as a 'scaffold' protein, coordinating signal inputs derived from receptors on the cell surface with intracellular signal pathways (Xie *et al*., 2006). As mentioned before, Dectin-1 has also been identified as an important partner for TLR2 on macrophages and dendritic cells for the production of inflammatory cytokines in response to certain stimuli like β-glucans (Gantner *et al*., 2003). TLRs do not recognize β-glucans on their own, but need Dectin-1 for this. All TLR use the adaptor protein MyD88 in their signalisation. When this protein is associated with the receptor, it recruits a family of IL-1 receptor associated kinases (IRAK), which take care of the phosphorylation and activation of TRAF-6. Further activation of kinases in this cascade leads to the release of NF-κB which will be tranlocated to the nucleus and subsequently mediates an elevated expression of inflammatory cytokines such as TNF-α, IL-6 and IL-12 (Yadav and Schorey, 2006). Signals from both TLR2 and Dectin-1 are required for the activation of NF-κB and the production of these cytokines. The generation of this response occurs on the cell surface and needs an intact ITAM motif of Dectin-1 as well. Dectin-1 ligation to TLR2 leads to phosphorylation of the ITAM-like signalling motif with subsequent signals which generate

phagocytosis and activation of NADPH oxidase, resulting in microbial killing (Yadav and Schorey, 2006). This collaboration between Dectin-1 and TLR2 provides a useful model for clearing the interaction mechanisms between many innate immunoreceptors involved in microbial recognition. Human DC-SIGN and his murine homologue SIGNR1 are members of the same family as Dectin-1 (Parent *et al.*, 2002). This protein can bind zymosan as well as the yeast *C. albicans* (Taylor *et al.*, 2004). SIGNR1 on itself has weak phagocytotic properties, so receptors like Dectin-1 are required for this process.

4.2. Immune activation pathway in invertebrates

Fungal β-glucans can induce all of the major anti-microbial immune mechanisms found in invertebrates, including the humoral, cellular and phenoloxidase responses (see Fig. 2.2). The majority of these responses rely on protease cascades which are initiated by PAMP recognition in the haemolymph. In most cases, the components of these cascades and the mechanism by which they are activated by the appropriate PRR is unknown. Activation of these cascades triggers responses either in the haemolymph, such as coagulation, or in immunocompetent cells, such as anti-microbial peptide secretion.

The recognition of β-glucans can also lead to phagocytosis by certain haemocytes (Brennan and Anderson, 2004). Many of these mechanisms have been studied in *Drosophila* (Brennan and Anderson, 2004), but a great deal of information, especially regarding β-glucan recognition, has also been obtained through more classical approaches in other organisms, including Anopheles sp., *Maduca sexta* and *Bombyx mori*.

β-glucans have been shown to induce at least two types of humoral responses in invertebrates, coagulation and anti-microbial peptide secretion. Although less well described in insects, the coagulation cascade in response to PAMPs has been defined in other arthropods, particularly the horseshoe crab, and is thought to function in wound healing and to restrict the movement of microbes (Theopold *et al.*, 2002; Muta and Iwanaga, 1996).

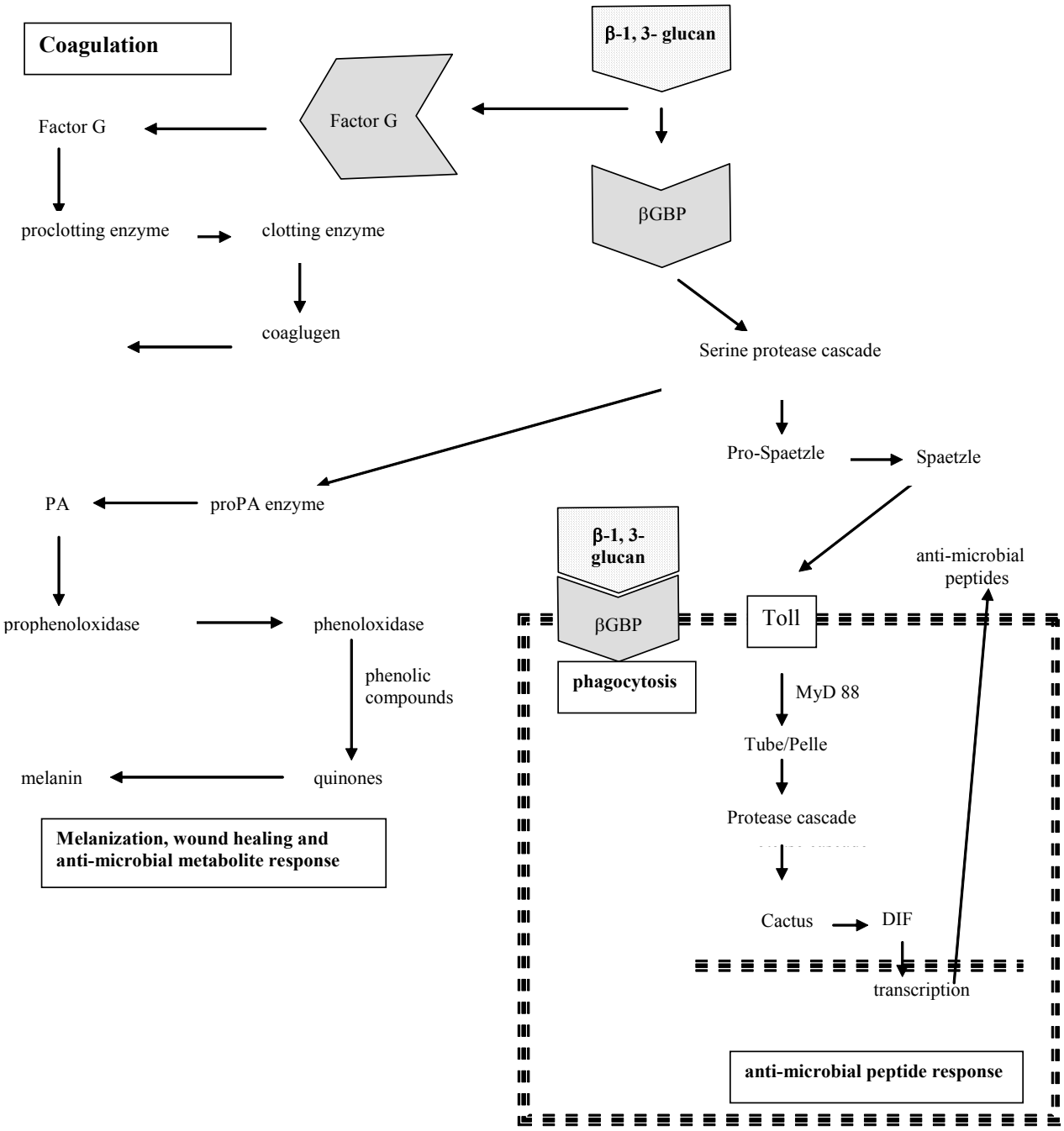

Fig. 2.2 β-glucan induced responses and protease cascades in invertebrates. The β-glucan receptors are highlited in grey. (PA, phenoloxidase activating enzyme) (modified from Brown and Gordon, 2005).

β-glucans in particular, are recognized by PRR, such as Factor G, which induces a series of sequential proteolysis events culminating in the coagulation of clottable proteins, such as coagulin. (The components of this cascade, which are released by haemocytes, are strictly regulated by protease inhibitors, such as serpins, and may be influenced by the phenoloxidase system (Muta and Iwanaga, 1996; Li *et al.*, 2002). Subsequent studies have revealed that, in contrast to the vertebrate receptors, invertebrate Toll receptors do not directly recognize PAMPs, but are rather part of the signalling pathway resulting in gene transcription and anti-microbial peptide production in the fat body (Hoffmann, 2003; Ferrandon *et al.*, 2004). The PRRs involved in sensing and triggering these pathways have only recently been identified, but many of the intermediate components remain unknown. Although the PRR(s) involved in β-glucan recognition via the Toll pathway have not been formally identified, they are likely to be members of the β-glucan recognition protein (βGRP) family (Ferrandon *et al.*, 2004). Recognition results in the production of peptides with anti-fungal activity, including Drosomycin, and may also trigger cellular responses (Hultmark, 2003). β-glucans recognition may also trigger the second, *imd/relish*, anti-microbial pathway, probably through recognition by membrane bound peptidoglycan recognition proteins (PGRPs) (Hedengren *et al.*, 1999; Hultmark, 2003).

To combat microbial infection, *Drosophila* activates multiple cellular and humoral responses including, for example, proteolytic cascades that lead to haemolymph coagulation and melanization, the production of several effector molecules such as antimicrobial peptides (AMPs) and the uptake of microorganisms by haemocytes (Tzou *et al*., 2002a). AMPs are made in the fat body, a functional equivalent of mammalian liver, and are secreted in the haemolymph, where they directly kill invading microorganisms (Hoffmann and Reichhart, 2002). Genetic analyses have shown that AMP genes are regulated by the Toll and Imd pathways (Hoffmann and Reichhart, 2002; Tzou *et al*., 2002a).

Upon infection, the Toll pathway is activated in the haemolymph by an uncharacterized serine protease cascade that involves the serpin Necrotic and leads to the processing of Spaetzle, the putative Toll ligand. Binding of Spaetzle to Toll activates an intracellular signalling cascade, involving the adaptor proteins dMyD88 and Tube, and the kinase Pelle, that leads to degradation of the Iκ-B-like protein Cactus and the nuclear translocation of the NF-κB-like transcription factors Dif and Dorsal (Hoffmann and Reichhart, 2002; Tzou *et al*., 2002a).

An extracellular recognition factor, peptidoglycan recognition protein (PGRP)-SA, belonging to a large family of proteins that bind to peptidoglycan has been implicated in the activation of the Toll pathway in response to Gram-positive bacteria but not fungi (β-glucans) (Michel *et al*., 2001). These data support the idea that the Toll pathway is activated by soluble recognition molecules that trigger distinct proteolytic cascades converging to Spaetzle. Recently, several studies have led to the genetic and molecular identification of seven components of the Imd pathway (Hoffmann and Reichhart, 2002; Tzou *et al*., 2002a). The ultimate target of the Imd pathway is Relish, a rel/NF-κB transactivator related to mammalian P105. Current models suggest that this protein needs to be processed in order to translocate to the nucleus. Its cleavage is dependent on both the caspase Dredd and the fly IκB−kinase (IKK) complex. Epistatic experiments suggest that dTAK1, a MAPKKK, functions upstream of the IKK complex and downstream of Imd, a protein with a death domain similar to that of mammalian receptor-interacting protein (Hoffmann and Reichhart, 2002; Tzou *et al*., 2002a). Recently, three independent studies have shown that a putative transmembrane protein, PGRP-LC acts upstream of Imd and probably functions in sensing microbial infection (Choe *et al*., 2002; Gottar *et al*., 2002; Rämet *et al*., 2002b).

Detailed knowledge about the role of PO (phenoloxidase) in invertebrate immunity is available for arthropods, notably insects and crustaceans. This availability is due to a long tradition of biochemical and more recent molecular studies of the proPO system in *Bombyx mori*, *Pacifastacus leniusculus*, *Holotrichia diomphalia*, and *Manduca sexta* (Cerenius and Söderhäll, 2004). Cellular

studies have been carried out in crustaceans, and several proteins that, in conjunction with the proPO system, are involved in encapsulation, phagocytosis, and cytotoxic reactions have been characterized. More recently, data have been gathered in non-arthropod systems, such as annelids (Bilej *et al.,* 2001), regarding proPO activation in conjunction with the presence of microbial products. It is well known that pathogenic microbial infections in insects and other invertebrates trigger the activation of the proPO system (Ashida and Brey, 1998). Several groups have determined the biological functions of proPO-activating factors and proPOs, suggesting that the proPO system is activated by a serine protease cascade and activated PO can synthesize melanin pigments for inhibiting spread of a microbial infection or for wound healing (Gillespie *et al.,* 1997; Ashida and Brey, 1998). However, a continuous activation of the proPO cascade, which could be harmful for the host organisms, might be well regulated by pattern recognition receptors, serpins, or proPO-activating factors. The early events of the proPO system consist of two parts; one is the recognition reaction between invading pathogens and pattern recognition proteins. The other is the signal transfer to the lower parts of the proPO system that a microbial invader is present in haemolymph. The biochemical link between the upstream and downstream parts of the proPO system is still unknown. It was reported that the 1,3-β-D-glucan pattern recognition protein is degraded by a serine protease(s) after recognition of non-self pathogens and after transfer of invasion signal to the downstream part of the proPO system (Ashida and Brey, 1998). Moreover, some PRR recognizing PGN (peptidoglycan) can also function to recognize β-1,3-glucan and induce the PPO cascade (Lee *et al*., 2004). In a preliminary study, the β-1,3-glucans isolated from yeast cell walls exhibited a significant stimulation of the PPO system activity in haemocytes *in vitro* and in haemolymph *in vivo* of black tiger shrimp, *Penaeus monodon* (Suphantharika *et al*., 2003).

Proliferation of cells involved in immune reactions in invertebrates

The hematopoietic tissue (hpt) has been detected and its morphology studied in several crustaceans, but the mechanisms behind the release of haemocytes into circulation in most

invertebrates are still unknown. Hpt is believed to contain precursor cells of the different haemocyte types. In crustaceans such as crayfish, the sheet-like hpt is located dorsally on the stomach and is particularly abundant in the hollow between cardiac and pyloric stomach. The hpt comprises small lobules containing differentiating and maturing haemocytes, is surrounded by a thin sheath of collagenous connective tissue and is in close contact with the haemolymph sinuses (reviewed in Johansson *et al.,* 2000). The hpt has different cell types based on their morphology. Hence, in crayfish *P. leniusculus*, hpt has at least five different types of cells which might correspond to developmental stages of granolucytes (GCs) and semi-granolucytes (SGCs). Type 1 was considered the least differentiated precursor cell while Types 2, 3 and 4 were speculated to be different stages of GCs development due to the presence of granules. Type 5, cells differ to Types 2–4 and was suggested to be a precursor of SGCs. According to Van de Braak *et al.* (2002), the SG cells of at least the large-granular cell line migrate and mature in the connective tissue. The connective tissue provides a reservoir of these G cells which can be easily mobilised at times of stress. The function of this redistribution of haemocytes may be interpreted in terms of enhancing the effectiveness of the internal defence system; when necessary, the animal is able to mobilise directly functionally active G cells. Moreover, some cells in the lobules of hpt show similar morphology with the circulating haemocytes. This suggests that the haemocytes are mature when they are released to the circulation (Chaga *et al.,* 1995). Similar cell types have been described in the hpt of lobster, *Homarus americanus* (Martin *et al.,* 1993), blue crab, *Carcinus sapidus* (Johnson, 1984) and shrimp, ridgeback prawn, *Sicyonia ingentis* (Hose *et al.,* 1992) and black tiger shrimp, *Peneus monodon* (Van de Braak *et al.,* 2002) although different synonyms were named for the different cell types. It is very likely there are no, or very few, haemocytes dividing after entering the circulation (Gargioni and Barracco, 1998; Sequeira *et al.,* 1996). The number of haemocytes in the circulating system, thus, is regulated by the hematopoiesis in hpt or by storage at other site. The circulating haemocytes of invertebrates are essential in immunity, performing functions such as phagocytosis, encapsulation and lysis of foreign cells (Söderhäll and Cerenius,

1998). The number of free haemocytes can vary and decrease dramatically during an infection (Söderhäll and Söderhäll , 2001). Thus new haemocytes need to be produced and released from hematopoietic tissues. Homeostasis seems to operate to restore haemocyte number in the haemolymph circulation of decapods (Smith and Söderhäll 1983, 1984; Lorenzon *et al.* 1999; Smith and Ratcliffe 1980). The mechanisms underlying the restoration of the haemocyte pool in crustaceans is not fully understood, but it is generally thought to entail either mobilisation of haemocytes from reservoirs in haemal crypts or upregulation of cell division in the haemopoietic tissue and proliferation within the haemolymph (Ghiretti-Magaldi *et al.,* 1977; Hose *et al.,* 1992). Hematopoiesis is the process by which haemocytes mature and subsequently enter the circulation. The formation and development of mature haemocytes involve proliferation, commitment and differentiation from undifferentiated hematopoietic cells. In fact, hematopoiesis provides a mechanism by which haemocytes that have expired or are damaged can be replaced by newly synthesized cells (Barreda and Belosevic, 2001; Medwinsky and Dzierzak, 1999). Haemocytes are constantly produced, although the rate by which this process occurs can be altered rapidly under the influence of different micro-environmental factors (Jiravanichpaisal *et al.,* 2006).

The hematopoietic process by which stem cells differentiate to produce erythroid, myeloid and lymphoid lineages has been widely studied in several vertebrate species, whereas equivalent processes in invertebrates are largely unknown (Söderhäll and Söderhäll, 2001).

In mammals, various types of blood cells have specific functions that are crucial for the survival of an individual, such as oxygen transport and defence against infection. In invertebrates, the most important role of the circulating haemocyte is the protection of the animal against invading microorganisms by participating in recognition, phagocytosis, melanization and cytotoxicity (Cerenius and Söderhäll, 2004; Tzou *et al.,* 2002). In Decapod crustaceans cellular immune reactions involve three types of circulating haemocytes; the hyaline cell (HC), the semi granular cell (SGC) and the granular cell (GC), which can be distinguished by their distinct appearance

(reviewed in Johansson *et al.,* 2000), although some synonymous haemocyte terms have been reported by others. This classification was mostly based on morphology.

In crayfish, a fungal infection can be mimicked by injection with a β-1,3-glucan such as laminarin, which will induce a rapid drop in haemocyte number followed by a recovery after 24–48 hours (Söderhäll *et al.,* 2003). The hpt of crayfish was found to be actively proliferating. Injection of β-1,3-glucan caused a severe loss of haemocytes resulting in an accelerated maturation of haemocyte precursors in the hpt followed by release into the circulation of new cells, which developed into functional SGCs and GCs expressing the proPO transcript. The loss of circulating haemocytes after β-1,3-glucan injection is probably due to cell aggregation inside the animal, indicating an important role of the haemocytes in defence. It was shown that in *Fusarium*-infected shrimp the rate of proliferating haemocytes significantly increased. In fact, haemocyte production seems to be required in fungal infection since the melanized lesions, which are characteristic of this *Fusarium* contamination, involve haemocyte waste (Persson 1987, Thomqvist, 1993). Therefore, fungal infection not only affects hemocytic behaviour as earlier described for freshwater crayfish (Persson 1987; Thomqvist, 1993), but also haemocyte proliferation and perhaps haemocyte synthesis or metabolism are affected.

According to a general agreement, circulating haemocytes of most crustaceans do not divide (Söderhäll and Cerenius, 1992) and, therefore, it was suggested that old cells must be continuously replenished by cells released into the haemolymph. However, in *Penaeus japonicus* 0·6% of the circulating haemocytes were in a proliferation stage and this increased to 3% after LPS injection or *Fusarium* infection (Sequeira *et al.,* 1996). These results demonstrate that the shrimp circulating haemocytes normally scarcely divide, but that proliferation increases after stimulation. It is speculated that it is mainly the younger cells, which are mature in the hpt, that still have the capability to proliferate.

An efficient immune system depends upon the interaction of many cellular and humoral components which develop at different rates during fetal and early postnatal life. Mostly the cells involved in the immune response are derived from hpt and are thought to differentiate into the various cell lineages under the influence of different micro environmental factors.

Transcription factors are key elements in lineage determination during haemocytes formation and several factors are conserved in *Drosophila* (Denk *et al.,* 2000; Fossett and Schultz, 2001; Lebestky *et al.,* 2000) and mammals (Busslinger *et al.,* 2000; Cumano and Godin, 2001). Recently, four genes; serpent (srp), lozenge (lz), U-shaped (Ush) and glial cell missing (gcm) have been found to be involved in *Drosophila* hematopoietic lineage commitment (Fossett and Schulz, 2001; Lebetsky *et al.,* 2000; Rehorn *et al.,* 1996). These genes, except gcm, have similarities to mammalian hematopoietic factors; GATA, Acute Myeloid Leukemia 1 (AML1) or Runx and Friend of GATA (FOG), respectively.

Several transcription factors and signaling pathways have been demonstrated to regulate haematopoietic lineage specification in *Drosophila* (reviewed by Meister, 2004). The GATA factor Serpent (Srp) confers haemocyte identity on the precursors in embryos, and probably also in the larval lymph gland as it is expressed before all differentiation markers in prohaemocytes. Proliferation is regulated by the PVF2/PVR, the Ras/Raf, and the JAK/STAT pathways. Crystal cell specification in larvae is under the control of Serrate/Notch pathway and the Runx1 homolog Lozeng transcription factor, and antagonized by the friend-of-GATA homolog U-shaped. Plasmatocytes are specified by two glial-cells-missing transactivators, and then they will further differentiate into pupal macrophages under the influence of the steroid hormone ecdysone. Lamellocyte specification probably involves the JAK/STAT pathway.

A PlRunt gene, encoding a Runt protein, which is involved in hematopoiesis in *Drosophila* and mammals, was upregulated prior to release of haemocytes into the haemolymph circulation. In contrast, proPO were expressed in these cells after they were released into the circulation

(Söderhäll *et al.,* 2003). Although during the past decades a number of studies of invertebrate transcription factors and signaling pathways regulating lineage commitment in hematopoietic development have been carried out, little is known about hematopoietic cytokines among invertebrates. Recently, it was reported that differentiation and growth of hematopoietic stem cells in vitro from crayfish, *P. leniusculus*, required an endogenous cytokine like-factor named astakine, which contains a prokineticin domain (Söderhäll *et al.,* 2005). Discovery of this first invertebrate prokineticin-like protein, which is homologous to prokineticins in many vertebrates, involved in hematopoiesis may provide interesting information concerning the evolution of growth factors and haemolymph development.

5. Application of β-glucans

5.1. Application of β-glucans in aquaculture

Diseases are still a major constraint to sustainable aquaculture production, especially for the farming of invertebrates (Bachère, 2003). In intensive systems, fish and shellfish species are often exposed to stressful conditions, eventually becoming more susceptible to microbial infections, especially in their larval stages (Smith *et al.,* 2003). The use of immunostimulants can improve innate defence of animals providing resistance to pathogens during periods of high stress, such as grading, reproduction, sea transfer and vaccination. Different kinds of substances are known to act as immunostimulants but only a few are suitable for use in aquaculture (Raa *et al.,* 1992; Siwicki *et al.,* 1998). Immunostimulating effects of glucans have a significant commercial potential. Several different types of β-glucan have been successfully used to enhance resistance of fish and crustaceans against bacterial and viral infections (Song *et al.,* 1997; Itami *et al.,* 1998; Chang *et al.,* 1999, 2000; Cook *et al.,* 2001; Paulsen *et al.,* 2001; Bagni *et al.,* 2005). It has been shown that β-glucans may improve health, growth and general performance of many different

animal groups, including farmed shrimp, fish and land animals. The immunomodulatory effects of glucans are not unequivocal and have been shown to be different in relation to the product source, animal species, development stage of the target organism, dose and type of glucan, route and time schedule of administration (Guselle *et al.,* 2007) and the association with other immunostimulants.

Many studies have measured the effects of glucan on immunity of fish (see Table 2.2). For instance, the *in vitro* culture of macrophages with glucan has been adopted by some authors (Cook *et al.,* 2001; Dalmo and Seljelid, 1995), but more were focused on *in vivo* studies (Robertsen *et al.,* 1990; Aakre *et al.,* 1994; Sahoo and Mukherjee, 2001; Ortuno *et al.,* 2002).

Table 2.2 The effects of β-glucans on fish and shrimp

AGENT	SOURCE	AUTHORS	FISH	ADMINISRATION	RESULTS	RESISTANCE TO PATHOGEN
curdlan	Bacteria *(Alcaligenes faecalis)*	**Lee *et al.* (2000)**	Crayfish	In vitro	PO activity ↑	
glucan	barley	Misra *et al.* (2006)	carp	oral	SGR ↑ FCR ↑ Immune parameters ↑	*Aeromonas hydrophila* ↑ *Edwardsiella tarda* ↑
glucan	mushroom *(Pleurotus florida)*	Kamilya *et al.* (2006)	carp	in vitro	Phagocytosis ↑ Bactericidal activity ↑ proliferative response ↑ NO↑, superoxide anion ↑ Hydrogen peroxide ↑	
glucan	mushroom (*Pleurotus florida*)	Kamilya *et al.* (2006)	carp	ip	MAF ↑ Antibody ↑	*Aeromonas hydrophila* ↑ *(adjuvant)*
Lentinan	mushroom (*Lentinus edodes)*	Yano *et al.* (1991)	carp	ip	phagocytosis ↑	*Edwardsiella tarda* ↑
Lentinan	mushroom (*Lentinus edodes)*	Figueras *et al.* (1998)	turbot	ip	phagocytosis ↑ haemolytic plaque ↑ agglutinating antibody ↑	*Vibrio damsela* ↑
Lentinan	mushroom (*Lentinus edodes)*	Nikl *et al.* (1991)	salmon	ip	• antibody ↑	*Aeromonas salmonicida* ↑ *(adjuvant)*
Chrysolaminaran	Marine diatom *(Chaetoceros Müleri)*	Skjermo *et al.* (2006)	Atlantic cod	Oral (via rotifers)	growth rate ↑ Survival ↑	
laminaran	brown algae *(Laminaria hyperborean)*	Dalmo *et al.* (1998)	salmon	oral • ip	Survival → Survival ↑	*Vibrio salmonicida* → *Vibrio salmonicida* ↑
laminaran	brown algae (*Laminaria hyperborean)*	Samuel *et al.* (1996)	blue gourami	ip	phagocytic activity ↑	*Aeromonas hydrophila* ↑
laminarin	algae*(Laminari digita)*	Magnadottir *et al.* (2006)	Cod larvae	Oral immersion	Survival →	*Aeromonas salmonicida* →
laminarin	*algae* (*Laminaria digita*)	Hellio *et al.* (2007)	oyster	In vitro	PO-like activity ↑	

Table 2.2 The effects of β-glucans on fish and shrimp (Continued)

AGENT	SOURCE	AUTHORS	FISH	ADMINISRATION	RESULTS	RESISTANCE TO PATHOGEN
laminarin	algae*(Laminari digita)*	Soltanian *et al.* (in press)	Artemia	immersion	Survival →	*V.campbellii* →
glucan	yeast *(Saccharomyces cerevisiae)*	Campa-Córdova *et al.* (2002)	shrimp	immersion	SOD activity ↑ THC ↑	*V. parahaemolyticus* ↑
glucan	yeast (*S. cerevisiae*)	Misra *et al.* (2004)	Shrimp (*Macrobrachium roosenbergii)*	immersion	lysozyme-like activity ↑ ACP ↑	*V. alginolyticus* ↑
glucan	yeast (*S. cerevisiae*)	Whittington *et al.* (2005)	tilapia	oral		*Streptococcus iniae* → *(adjuvant)*
glucan	yeast (*S. cerevisiae*)	Selvaraj *et al.* (2005)	carp	ip	antibody ↑	*Aeromonas hydrophila* ↑
glucan	yeast (*S. cerevisiae*)	Marques *et al.* (2006)	Artemia	immersion	Survival ↑	*V.campbellii* ↑
glucan	yeast (*S. cerevisiae*)	Kumari and Sahoo (2006)	catfish	oral	antibody ↑ disease resistance ↑	*Aeromonas hydrophila* ↑
glucan	yeast (*S. cerevisiae*)	Guselle *et al.* (2006)	trout	ip	xenoma formation ↓	*Loma salmonae* ↑
Zymosan	yeast (*S. cerevisiae*)	Ching-Yi *et al.* (2005)	shrimp	In vitro	PO activity ↑	
Zymosan	yeast (*S. cerevisiae*)	Zhang *et al.* (2005)	shrimp	injection	Hematopoiesis ↑ SOD ↑, ALP ↑, ACP ↑, PO ↑	
Zymosan	yeast (*S. cerevisiae*)	Hellio *et al.* (2007)	oyster	In vitro	PO-like activity ↑	
Zymosan	yeast (*S. cerevisiae*)	Soltanian *et al.* (in press)	Artemia	immersion	Survival ↑	*V.campbellii* ↑
Zymosan	yeast (*S. cerevisiae*)	Buggé *et al.* (in press)	clam	In vitro	ROS ↑	
MacroGard	yeast (*S. cerevisiae*)	Siwicki *et al.* (1994)	Turbot	oral	myeloperoxidase activity ↑ phagocytosis ↑	*Aeromonas Salmonicida* ↑
MacroGard	yeast (*S. cerevisiae*)	Supamattaya *et al.* (2000)	shrimp	immersion	Growth performance ↑ Survival ↑	
MacroGard® Fibosel® VitaStim®	yeast *(S. cerevisiae)* yeast (*S. cerevisiae*) *yeast (Schizophyllum commune)*	Couso *et al.* (2003)	seabream	oral	phagocytosis → respiratory burst →	*Photobacterium damselae*↑

Table 2.2 The effects of β-glucans on fish and shrimp (Continued)

AGENT	SOURCE	AUTHORS	FISH	ADMINISRATION	RESULTS	RESISTANCE TO PATHOGEN
MacroGard	yeast (*S. cerevisiae*)	Bagni *et al.* (2005)	Sea bass	oral	complement activity ↑ lysozyme ↑	
MacroGard	yeast (*S. cerevisiae*)	Palić *et al.* (2006)	Fish (fathead minnows)	In vitro oral	Neutrophil activity ↑	
MacroGard	yeast (*S. cerevisiae*)	Soltanian *et al.* (in press)	Artemia	immersion	Survival ↑	*V.campbellii* ↑
EcoActiva	yeast (*S. cerevisiae*)	Cook *et al.* (2003)	Fish (snapper)	oral	macrophage respiratory burst ↑ growth rate ↑	
schizophyllan sceroglucan lentinan	*S. commune* *S. glucanicum* (yeast) *L. edodes* (mushroom)	Yano *et al.* (1989)	carp	ip	phagocytosis ↑	*Edwardsiella tarda* ↑
Schizophyllan Scleroglucan	*S. commune* *Sclerotium glucanicum*	Matsuyama *et al.* (1992)	Fish (yellowtail)	ip	Complement ↑ Lysozyme ↑ Phagocytic index ↑	*Streptococcus* sp. ↑ *Pasteurella piscicida* →
Schizophyllan	*S. commune*	Itami *et al.* (1994)	prawn	oral	phagocytosis ↑	*Vibrio sp.* ↑
Schizophyllan	*S. commune*	Chang *et al.* (2000)	*Penaeus monodon*	oral	phagocytosis ↑	
Schizophyllan	*S. commune*	Chang *et al.* (2003)	prawn	oral	PO activity ↑ SOD activity ↑ O_2^- ↑	WSSV ↑
Scleroglucan	*S. glucanicum*	Yano *et al.* (1991)	carp	ip		*Aeromonas hydrophila* ↑

Fish recognize β-glucans as foreign agents because of their similarity to fungal or bacterial Gram-negative polysaccharides. After exposure, the immune system of fish produces an inflammatory response, as it would be against a pathogen, which provides effective protection against the opportunistic pathogens (Robertsen *et al.,* 1994). Numerous studies have reported that β-glucans induce an increase in the resistance of fish to several bacterial pathogens through an increase in the levels of complement and lysozyme as well as an enhancement of the phagocytic, respiratory burst and bactericidal activities of fish phagocytes (Robertsen *et al.,* 1994; Dalmo *et al.,* 1996; Figueras *et al.,* 1998). Different administration routes (oral, immersion, injection) have produced different results. β-glucans are usually given by intraperitoneal injection because of efficiency and quickness of the method (Robertsen *et al.,* 1990; Chen and Ainsworth, 1992; Matsuyama *et al.,* 1992; Jørgensen *et al.,* 1993). The drawback of injection is that it is labor intensive and also stressful to fish. Bathing and oral administration are potentially useful alternative methods for mass administration to fish of all sizes. However, there is very little information available on bathing and oral administration protocols or dosage in farmed fish species. Robertsen and coworkers (1990) have also stated that the protection obtained by oral administration may often be relatively low compared to injection and the individual variation in response to immunostimulants may be large. Furthermore, the degradation and absorption of glucan in the digestive tract after oral administration must be taken into account, as this may modify its effect on the immune system (Ortuno *et al.,* 2002).

Some reports suggest that β-glucans enhance the protection of fish against bacterial or protozoan infections when administered in the feed (Nikl *et al.,* 1993; Siwicki *et al.,* 1994; Yoshi *et al.,* 1995; Efthimou, 1996). However, there are also reports which state that no protection of fish has been obtained in such experiments (Toranjo *et al.,* 1995; Baulny *et al.,* 1996). It was reported that long-term oral administration of peptido-glucans decreased the immune response in rainbow trout when challenged with *Vibrio anguillarum* (Matzuo and Miyazono, 1995), as well as in catfish (Yoshida *et al.,* 1995), suggesting a negative feedback effect of β-glucan (Sakai, 1999). Some authors

mentioned that a high level of β-1,3-glucan directly induced the respiratory burst, which, after a period, can exhaust the immune cells resulting in immunosuppression or feedback regulation (Ai *et al.,* 2007; Castro *et al.,* 1999; Robertsen *et al.,* 1994). There is still little information on how long the defence mechanism can be immunostimulated. Most studies have only looked at short-term protection for 1–2 weeks, which is marked upon administration of immunostimulants such as β-glucan and chitosan (Anderson and Siwicki 1994). It was reported that multiple injections of β-glucan (at two-week intervals) might have maintained the activation of phagocytic cells for a longer period, which in turn would lead to long-term protection in fishes. Administration of different forms of β-glucan in the diet of different shrimp species has also resulted in enhancement of protection against various pathogens (Song *et al.,* 1997; Itami *et al.,* 1998; Chang *et al.,* 1999, 2000). This increased resistance is in part due to the enhancement of phagocytic activity of haemocytes (Itami *et al.,* 1994). During phagocytosis, a series of microbicidal substances including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) hydroxide ions (OH^-), singlet oxygen (O_2^1), myeloperoxidase (MPO) catalysed hypochlorites and various lysosomal enzymes are generated to kill the invading pathogen (Segal, 1989).

A relatively small number of reports have addressed the issue of immunostimulation for the larval stages although several reports highlight the larval and post-larval stages as those most susceptible to disease. Unfortunately, we know very little about the ontogeny and early defence mechanisms of the immune system in aquatic invertebrates and fish larvae as in general their small size precludes direct study. It cannot be assumed that juveniles exhibit the same responses as adults, or that the expression of immune proteins occurs to the same degree. In fact, the impact of immunostimulants on the developing immune system of larval fish is not clear. Some researcher maintain that the effect is minimal and immunostimulants can be fed to larval fish as soon as the animal can be weaned to an artificial diet (Bricknell and Dalmo, 2005). However, it is also believed that administering potentially powerful immunomodulating compounds to an animal that has still to

undergo major developmental changes in the immune system could result in induction of tolerance against immunostimulants. In fish, immunocompetence is achieved some time after the formation of the lymphomyeloid organs and appearance of the different leukocyte populations (Lam *et al.,* 2004, Huttenhuis, 2005). Fish immunocompetence is determined by the functioning of lymphocytes rather than by the histological development of lymphoid organs or the morphological identification of lymphoid cells. The exact timing of lymphocyte differentiation varies in different fish species and is probably related to the rate of growth which is strongly affected by culture conditions such as temperature, salinity and photoperiod, (Falk-Peterson, 2005) and general development. In penaeid shrimp the immune capability of larvae was studied during larval development. The expression and localization of penaeidin (Litvan-Pen3-1) have been studied in the first larval stages, from Nauplius V, Zoea I, II, III, to Mysis II, and in post-larvae (Munoz *et al.,* 2003). The results showed Litvan-Pen3-1 transcript and peptides are restricted to some haemocytes observed in the Mysis II larvae stage. This suggests either a low level of transcriptional activity in expressing cells, or a low number of expressing cells present in the larvae (Munoz *et al.,* 2003). In the marine mussel, *Mytilus edulis,* the levels of phagocytosis in immature larval haemocytes (e.g. in trochophore and veliger cells) was much lower (in either percentage of phagocytic cells or mean number of bacteria ingested per cell) in respect to adult mussel haemocytes (Munoz *et al.,* 2003).

Further functional studies are needed to ascertain about maturation of the immune system in different species before any efficient vaccination or immunostimulation protocol can be developed in order to be able to induce immunization rather than tolerance.

β-glucans are also used as helper substances (adjuvants) for fish bacterial vaccines (Nikl *et al.,* 1991; Nicoletti *et al.,* 1992; Anderson, 1992; Chen and Ainsworth, 1992; Rørstad *et al.,* 1993; Aakre *et al.,* 1994; Raa *et al.,* 1996; Ogier de Baulny *et al*., 1996; Figueras *et al.,* 1998; Selvaraj *et al.,* 2005; Kamilya *et al.,* 2006) (see also Table 2.2). The enhanced efficacy of vaccines after the administration of β-glucan is mediated through the modulation of host defences by increasing total

leucocytes, differential count (neutrophils, monocytes, lymphocytes, basophils and eosiniphils) (Selvaraj *et al.,* 2005) and immune parameters such as bacterial killing activity, phagocytosis (Sakai, 1999, Robertsen, 1999), production of anti-microbial mediators including superoxide anion (Sakai, 1999 and Robertsen, 1999), interleukin-1β (Selvaraj *et al.,* 2005), complement activity (Engstad *et al.,* 1992 and Verlhac *et al.,* 1996), activation of antigen-presenting cells (*e.g.* macrophages) (Raa, 2000) and also activation of lymphocytes (B cells) which produce specific antibody *i.e* in brown trout, eels, catfish and carp (Chen and Ainsworth, 1992; Raa 2002; Selvaraj *et al.,* 2005). In fish vaccination, adjuvants are usually administered simultaneously with the vaccine. Some authors (Anderson *et al.,* 1989) have demonstrated that if the vaccine is administered after the immunostimulant, the immune response is greatly reduced, mainly at high doses. However, the group of Figueras *et al.,* (1998) reported the highest activity of all the immune parameters when glucans were injected after the bacterin. In another study, the authors (Chen and Ainsworth, 1992) reported higher antibody levels when the glucans were administered combined with the antigen and were significantly reduced when they were administered before the bacterin. It is concluded that the sequence of glucan administration is critical when used as a vaccine adjuvant.

Application of adjuvanted vaccine is a good strategy as the successful development of new vaccines is reliant upon the availability of adjuvants that are not only safe for the host, but also induce immune responses complementary to those generated during natural infection (Actor *et al*. , 2002).

5.2. Application of β-glucans in vertebrates

As mentioned before, β-glucans have been extensively used to improve health, growth and general performance in many different animal groups, including shrimp, fish, but also in land animals (see Table 2.2 and 2.3)

Table 2.3 β-glucans and their application in vertebrates (*in vivo* and *in vitro*)

AGENT	SOURCE	AUTHORS	VERTEBRATE	ADMINISTRATION	RESULTS	RESISTANCE TO PATHOGEN
AG	Algae *(Euglena gracilis)*	Mohagheghpour *et al.*, 1995	Mice	IP	Antibody titers, T cell proliferation, IL-2 production↑	
AG	Algae *(Euglena gracilis)*	Mohagheghpour *et al.*, 1995	Rabbit	IP	Antibody titers↑	
β-glucan	Yeast cell wall (soluble)	Xiao *et al.*, 2004	Pig	*In vitro*	Cellular and PRRSV-specfic immune response↑	PRRSV
β-glucan	Yeast (*S. cerevisiae)*	Li *et al.*, 2005	Pig	Oral	In vivo ↑ IL-6, TNF-α and IL-10	LPS
β-glucan		Hahn *et al.*, 2006	Pig	Oral	Antiboy titers against ag↑, CD4 and CD8 cells↑	
β-glucan		Lowry *et al.*, 2005	Chicken	Oral	Phagocytosis, bacterial killing and respiratory burst↑	*Salmonella enterica* serovar enteritidis
β-1,3-glucan	Yeast	Engstad *et al.*, 2002	Human	*In vitro*	IL-8, TF ↑↑, TNF-alpha, IL-6, IL-10 ↑, synergistic to LPS, neutrophil degranulation	
β-1,3-glucan WGP	Yeast	Vetvicka *et al.*, 2002	Mice	Oral	TNF-alpha, IL-1beta↑	*Bacillus anthracis* (Anthrax)
β-1,3-glucan	Yeast	Waller and Colditz, 1999	Sheep	Intramammary	Monocyt/macrophage number↑	
β-1,3-D-glucan	Yeast (*Candida albicans)*	Yoshida *et al.*, 1996	Rabbit	IV		
β-1,3-glucan		Persson Waller *et al.*, 2003	Cattle	Intramammary	MHCII+ lymphocytes↑	*Staphylococcus aureus*
β-1,3-glucan (insoluble)	Yeast (*S. cerevisiae)*	Reynolds *et al.*, 1980	Mice	IV	Adjuvant effect vaccine, host resistance against challenge	Venezuelan equine encephalomyelitis
				IN		*Pseudomonas pseudomallei*
β-1,3-glucan (insoluble)	Yeast (*S. cerevisiae)*	Reynolds *et al.*, 1980	Rat	IV	host resistance against challenge	*Francisella tularensis*
β-1,3-glucan (insoluble)	Yeast (*S. cerevisiae)*	Reynolds *et al.*, 1980	Monkey	IV	VEE antibody titers↑	
β-1,3/1,6-glucan	Yeast (*S. cerevisiae)*	Williams and Di Luzio, 1979	Mice	IV	Leukocyte number, microbicidal activity↑	*Staphylococcus aureus*
β-1,3/1,6-glucan	Yeast (*S. cerevisiae)*	Lee *et al.*, 2002	Human	*In vitro*	TNF-alpha↑, stimulates macrophage function	
β-1,3/1,6-glucan	Yeast (*S. cerevisiae)*	Tsukada *et al.*, 2003	Mice	Oral	IEL↑, IFNgamma↑	
β-1,3/1,6-glucan	Yeast (*S. cerevisiae)*	Hiss and Sauerwein, 2003	Pig	Oral	Growth performance↑	
β-1,3/1,6-glucan	Yeast ?	Guo *et al.*, 2003	Chicken		IL-1, nitrite↑, proliferation mΦ ↑, lymphocytes↑, lymphoid organs larger	
β-1,4-glucan	Bacteria *(Acetobacter*	Li *et al.*, 2004	Mice	IG	Survival rate ↑, CD4+ and CD8+ cells ↑,	*Listeria monocytogenes*

Glucan-P	Yeast (*S. cerevisiae*)	Rice *et al.*, 2005	Mice	Oral	Systemic IL-12↑	*Staphylococcus aureus* *Candida albicans*
Krestin (PSK)	Fungus *(Coriolus versicolor)*	Tochikura *et al.*, 1987	Human	In vitro	Block cytopathic effect virus	HIV
Krestin (PSK)	Fungus *(Coriolus versicolor)*	Asai *et al.*, 2005	Mice	*In vitro*	IL-6↓, NO production↓ MΦ Protection against LPS lethality	
Krestin (PSK)	Fungus *(Coriolus versicolor)*	Sakagami *et al.*, 1991	Mice Human	IV *In vitro*	MΦ: NBT reducing↑ Human PMN: iodination↑ TNF↑	
Laminarin	Algae *(Laminaria digitata)*	Rice *et al.*, 2005	Mice	Oral	Systemic IL-12↑	*Staphylococcus aureus* *Candida albicans*
Lentinan	Fungus (*Lentinus edodus)*	Irinoda *et al.*, 1992	Mice	IN IV	NO and CL activity↑ alveolar MΦ, IL-6 production	Influenza
Lentinan	Fungus (*Lentinus edodus)*	Kupfahl *et al.*, 2006	Mice	*In vitro* IV	TNF-alfa , IFN-gamma and IL-12↑, NO produc-tion and cytotoxic, CD8↑	*Listeria monocytogenes*
MacroGard	Yeast (*S. cerevisiae)*	Decuypere *et al.*, 1998	Pig	Oral	Immunoreaction to vaccination↑	
MacroGard	Yeast (*S. cerevisiae)*	Instanes *et al.*, 2004	Mice	SC	Adjuvant effect toward OVA, on allergic responses	
ObG	Oat	Yun *et al.*, 2003	Mice	IG IP	*In vitro* phagocytosis and proliferation mΦ↑ Induction specific IgG/IgA Cytokine secretion↑	*Staphylococcus aureus* *Eimeria vermiformis*
PGG	Yeast (*S. cerevisiae)*	Wakshull *et al.*, 1999	Human	*In vitro*	Oxidative burst, leukocyte microbicidal activity↑	
PGG	Yeast (*S. cerevisiae)*	Onderdonk *et al.*, 1992	Rat	IV	Leucocyte number↑	*Escherichia coli,* *Staphylococus aureus*
PGG	Yeast (*S. cerevisiae)*	Onderdonk *et al.*, 1992	Mice	IV	Leucocyte numbe↑	*Escherichia coli,* *Staphylococus aureus*
PGG (Betafectin)	Yeast (*S. cerevisiae)*	Liang *et al.*, 1998	Rat	IM	Monocyt, neutrophil number↑, oxidative activity↑	*Staphylococcus aureus*
PGG (Betafectin)	Yeast (*S. cerevisiae)*	Kernodle *et al.*, 1997	Guinea pig	IV	Neutrophil activation Dose-effect response wound infection	*Staphylococcus aureus* *Staphyococcus epidermidis*
Scleroglucan	Yeast (*S. cerevisiae*	Rice *et al.*, 2005	Mice	Oral	Systemic IL-12↑	*Staphylococcus aureus* *Candida albicans*
SSG	Fungus (*Sclerotinia sclerotiorum*)	Suzuki *et al.*, 2001	Mice	IP	IgG2a, IFN-gamma, IL-12↑	
SSG	Fungus (*Sclerotinia sclerotiorum*)	Hetland *et al.*, 2000	Mice	IP	Protective and curative against pneumococcal infection	*Streptococcus pneumoniae*
TLFN, 1,3/1,6-glucan	Yeast (*S. cerevisiae)*	Krakowski *et al.*, 1999	Horse	IM	Activity PMN↑ Concentration IgG/IgM colostrum↑	

One important aspect of the immunobiological activity of β-glucans will be their adjuvant effect. In general the immune system reacts with a better and higher response when β-glucans 'prime' this system. Many studies have pointed out the possibility of β-glucans as an adjuvant in combination with viral, bacterial and parasitic vaccines (Hetland *et al*., 2000; Hrckova and Velebny, 2001; Yun *et al.*, 2003; Jung *et al.*, 2004). Glucans raise the humoral (Sakurai *et al*., 1992) as well as the cellular immunity (Xiao *et al.*, 2004) against these antigens. Moreover, glucans have an additional or even a synergistic effect on the immunity in combination with a variety of agents (Reynolds *et al.*, 1980; Williams *et al.*, 1999; Instanes *et al*., 2004) and even lead to resistance against viral (Irinoda *et al.*, 1992; Xiao *et al.*, 2004), bacterial (Kernodle *et al.*, 1998; Yun *et al.*, 2003; Lowry *et al.*, 2005) and parasitic (Yun *et al.*, 2003) pathogens (see table β-glucans and vertebrates). β-1,3-1,6-glucans also function as an adjuvant for an antitumor monoclonal antibody by priming CR3 on granulocytes (Yan *et al.,* 1999; Cheung *et al.*, 2002; Hong *et al.*, 2004). A discrete dosis related effect of β-glucans in the diet has been found on growth performance and resistance against infection in pigs. β-glucans can enhance the concentration of the IL-1 receptor antagonist. As a consequence the immune system is less activated, which results in a higher feed intake and growth performance. However, a resting immune system will lead to a higher sensitivity to challenge (Dritz *et al.*, 1995).

Clinical trials show anti-tumor activity of β-glucans, but only significant in the early stages of the cancer (Borchers *et al.*, 1999; Yalin *et al.*, 2005). The survival of these patients was prolonged and also their quality of life. The former was probably caused by the higher filtration of T- and B-lymphocytes and macrophages into the tumour, by prevention of metastasis and by the repair of the ratio Th1/Th2. *In vitro* studies also exerted a direct cytostatic effect on sarcoma and melanoma cells from particulate β-glucans (Williams *et al*., 1985; Okamura *et al.*, 1986, Tsang *et al.*, 2003; Nakano *et al.*, 1999; Tari *et al.*, 1994; Yoshino *et al.*, 2000). Alternative anti-tumor applications are combination therapies like β-glucans and cyclophosphamide in mice, resulting in the reduction of hepatic metastases and a prolonged survival rate. Especially mushroom β-glucans like lentinan,

krestin and schizophyllan have been proven to have anti-tumor activities, mostly in combination with chemotherapy or surgical removement of the primary tumour (Kimura *et al.*, 2003; Wasser, 2002). Studies evaluating the molecular weight dependence of antitumor activity have suggested that the triple-helix form is the most potent conformer of schizophyllan (SPG). However, other studies, using solid state ^{13}C NMR spectroscopy, have suggested that for antitumor activity and the production of tumor necrosis factor, nitric oxide and hydrogen peroxide by macrophage, the single-helix is the potent conformer.

Uptake of soluble β-glucans improves the lipid pattern of humans and laboratory animals with a high cholesterol amount (Robbins and Steeley, 1977). Clinical trials proved this effect on hypercholesterolemia with pure β-glucans from yeast and cereals in the diet (Anderson J.W. and Bridges S.R., 1993). The more fluid the diet, the more effect was seen from the β-glucans (Naumann *et al.*, 2006). In a study of Varady and Jones (2005) the effect of oat products were tested on persons with mild hypercholesterolemia. Results show significant reduction in total and LDL cholesterol, but no effect on HDL or triglycerides. One possible mechanism mentioned, is the induction of a higher viscosity in the intestine and thereby a reduction in the absorption of bile salts. In another study, krestin prevented lipoperoxidative damage and atherosclerotic plaques in rabbits, fed a high cholesterol diet (Pang, 2003). The prevention of this process of atherosclerosis is very important since this creates heart and vascular diseases, considerable causes of death. Some β-glucans (gel-forming) are used as feed additives to improve the physical properties as thickeners, fat replacers, water retaining agents or emulsifying stabilizers. Moreover, β-glucans from oat and barley can be used as functional feed ingredients, but more research about their health promoting function is necessary for the development of new applications in feed industry (Thammakiti *et al.*, 2004). When β-glucans are applicated through feed, they can activate the immune system (see above), but also have a positive effect on the cholesterol (see above) and on the insulin and glucose amount in diabetic patients (Brennan and Cleary, 2005).

Few studies have reported that β-glucans can adsorb particular mycotoxins, thereby preventing their harmful effect (Devegowda *et al.*, 1998; Dawson *et al*., 2001; Yiannikouris *et al.*, 2004;). This binding is dosis dependent and reversible. *In vitro* studies have demonstrated this effect with a range of toxins and the cell wall of *Saccharomyces cerevisiae* (Devegowda *et al.*, 1998; Yiannikouris *et al.*, 2004).

Because of all these properties of β-1,3-glucans, several alternative medicines have turned up based on these carbohydrates (Brown and Gordon, 2003). Nevertheless, as mentioned above, their solubility in aquatic media is a major obstacle for the clinical use of β-glucans as biological response modifiers (Bohn and BeMiller, 1995).

Conclusion

Two different reasons for the studies of β-glucan in invertebrates exist: one is the general progress of our knowledge of the basic defense reactions in invertebrates, including phagocytosis, lectins or phenoloxidase systems; the other being the ever increasing need to find more environmental-friendly treatments for invertebrates (in contrast to chemical anti-microbial products) stressed by extensive farming and disease outbreak (*e.g.* crustacean larvae culture). The current evolution in feed industry is mainly focused on health promoters, higher feed efficiency and alternatives to antibiotics. Beside the prebiotic effects, β-glucans also have immunomodulating activities and may therefore come up to all of these properties. So, many studies focus now on the exact mechanism of these glucans and also further on their applications.

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CHAPTER III

Influence of different yeast cell-wall mutants on performance and protection against pathogenic bacteria (*Vibrio campbellii*) in gnotobiotically-grown *Artemia*

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Abstract

A selection of isogenic yeast strains (with deletion for genes involved in cell-wall synthesis) was used to evaluate their nutritional and pathogen-protective properties for gnotobiotically-grown *Artemia.* In the first set of experiments the nutritional value of isogenic yeast strains (effected in mannoproteins, glucan, chitin and cell-wall bound protein synthesis) for gnotobiotically-grown *Artemia* was studied. Yeast cell-wall mutants were always better feed for *Artemia* than the isogenic wild type mainly because they supported a higher survival but not a stronger individual growth. The difference in *Artemia* performance between WT and mutants feeding was reduced when stationary-phase grown cells were used. These results suggest that any mutation affecting the yeast cell-wall make-up is sufficient to improve the digestibility for *Artemia.* The second set of experiments, investigates the use of a small amount of yeast cells in gnotobiotic *Artemia* to overcome pathogenicity of *Vibrio campbellii* (VC). Among all yeast cell strains used in this study, only mnn9 yeast (less cell wall-bond mannoproteins and more glucan and chitin) seems to completely protect *Artemia* against the pathogen. Incomplete protection against the pathogen was obtained by the gas1 and chs3 mutants, which are lacking the gene for respectively a particular cell wall protein and chitin synthesis, resulting in more glucan. The result with the chs3 mutant is of particular interest, as its nutritional value for *Artemia* is comparable to the wild type. Hence, only with the chs3 strain, in contrast to the gas1 or mnn9 strains, the temporary protection to VC is not concomitant with a better growth performance under unchallenged conditions, suggesting non-interference of general nutritional effects.

1. Introduction

Immunomodulation of larval fish has been proposed as a potential method for improving larval survival by increasing the innate responses of the developing animals until its adaptive immune response is sufficiently developed to increase an effective response to the pathogen (Bricknell and Damo, 2005).

Invertebrates are not equipped with cells that are analogous to antibody producing lymphocytes in vertebrates. According to Raa (2000), invertebrates are apparently entirely dependent on non-specific immune mechanisms to cope with infections, as they lack the specific immunological "memory" that is found in fish and warm-blooded animals. As a result, it does not seem to make sense to vaccinate them against a specific diseases .Yet, a recent study in the copepod *Macrocyclops albidus* showed that the defence system of this invertebrate species reacted more efficiently after a previous encounter with an antigenically similar parasite, implying that a specific memory may exist (Kurtz and Franz, 2003). Furthermore, exposure of shrimp to inactivated *Vibrio* spp. has been reported to provide some protection (Alabi *et al.,* 1999; Itami *et al.,* 1998; Teunissen *et al.,* 1998). The use of specific biological compounds (immunostimulants) that enhance immune responses of target organisms, rendering animals more resistant to diseases may be an excellent preventive tool against pathogens (Anderson, 1992). Such substances may reduce the risk of disease outbreaks if administered prior to a situation known to result in stress and impaired general performance (*e.g.* handling stress, change of temperature or other environmental parameters, weaning from live to artificial feeds) or prior to an expected increase in exposure to pathogenic micro-organisms and parasites (*e.g.* spring and autumn blooms in marine environment, transfer to engrowing systems).

Several immunostimulants have been used in vertebrate and invertebrate culture, to induce protection against a wide range of diseases: *i.e.* β-glucans (Sung *et al.,* 1996; Sritunyalucksana *et al.,* 1999; Burgents *et al.,* 2004; Misra *et al.,* 2004), chitin (Anderson and Siwicki, 1994; Song and Huang, 1999; Wang and Chen, 2005), mannoproteins (Tizard *et al.,* 1989), lipopolysaccharides (Takahashi *et al.,*

2000), peptidoglycans (Itami *et al.,* 1998; Boonyaratpalin *et al.,* 1995) and dead bacteria (Alabi *et al.,* 1999; Keith *et al.,* 1992; Vici *et al.,* 2000).

Marques *et al*. (2004a,b) have recently developed and validated the usefulness of an *Artemia* gnotobiotic test system allowing to study the effect of food composition on survival and growth in the presence or absence of a pathogen. Baker's yeast *Saccharomyces cerevisiae,* which has been found to be a good immune enhancer in some aquatic organism, is an excellent source of β-glucans and chitin. These compounds together with mannoproteines constitute the major compounds of the yeast cell-wall (Magnelli *et al.,* 2002). The present study aims to identify the critical cell-wall components that induce pathogen-protection in *Artemia*. The effect of isogenic yeast deletion mutants (8 strains), carrying a null mutation in a gene involved in cell wall synthesis, was evaluated in a gnotobiotic *Artemia* test system. Firstly, *Artemia* performance was examined with the null-mutant yeast cells, harvested in exponential and/or stationary growth phase. In a second stage, these feed sources were tested in combination with a *Vibrio campbellii* challenge.

2. Methodology

2.1. Axenic culture of yeast

To verify the digestibility of live baker's yeast (*S. cerevisiae*) by *Artemia,* 7 different null-mutants of yeast (isogenic deletion strains derived from baker's yeast strain BY 4741) and the wild type strain (WT) (genotype described in Table 3.1) were fed to *Artemia*. All strains were provided by EUROSCARF (University of Frankfurt, Germany).

Yeasts cultures were performed according to procedures previously described by Marques *et al.* (2004a,b), using minimal Yeast Nitrogen Base culture medium (YNB).

Yeasts were harvested by centrifugation (± *800* × *g* for 10min), either in the exponential growth phase (after 20 h; "exp.yeast") or in the stationary growth phase (after 3 days;"stat.yeast"). Yeast cell concentrations were determined with a Bürker haemocytometer. Yeast suspensions were stored at 4 °C until the end of each experiment (maximum storage of one week).

• **Table 3.1** Genotype of all yeast strains used as feed for *Artemia* and description of each gene mutation in the development of cell wall components.

Strains	Genotype	Phenotype (cell wall changes)	Reference
WT	BY 4741*; Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0	control yeast	Dallies et al.(1998); Klis et al.(2002); Magnelli et al.(2002); Marques et al.(2004)
mnn9	BY4741; *Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0;YPL050c::kanMX4	less mannan, higher chitin, higher β- glucans	Klis et al.(2002); Klis et al.(2002); Magnelli et al.(2002); Marques et al.(2004)
mnn6	BY4741; *Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0;YPL053c::kanMX4	less phosphomannan	Karson and Ballou (1978); Wang et al (1997) Jigami and Odani (1999)
fks1	BY4741; *Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0;YLR342w::kanMX4	less β1,3-glucans, higher chitin	Dallies et al.(1998); Magnelli et al.(2002); Martin-Yken et al.(2002); Pagé et al.(2003) Aguillar-Uscana and Francois (2003)
knr4	BY4741; *Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0;YGR229c::kanMX4	less β1,3-glucans, higher chitin	Dallies et al.(1998); Magnelli et al.(2002); Martin-Yken et al.(2002); Pagé et al.(2003); Aguillar-Uscana and Francois (2003)
kre6	BY4741; *Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0;YPR159w::kanMX4	less β1,6-glucans, higher chitin	Magnelli et al.(2002); Francois(2003) ; Martin-Yken et al.(2002); Pagé et al.(2003)
chs3	BY4741; *Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0;YBR023c::kanMX4	less chitin	Valdivieso et al.(2000); Cabib et al.(2001); Klis et al.(2002); Magnelli et al.(2002);
gas1	BY4741; *Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0;YMR307w::kanMX4 **less β1,3- glucans, higher chitin**	less integration of yeast cell adhesion proteins into the cell wall	De Nobel et al.(1994); Popolo et al.(1997) Lipke and Ovalle (1998); **Magnelli et al.(2002)**

2.2. Bacterial strains and growth conditions

Two bacterial strains were selected, *i.e. Aeromonas hydrophila* strain LVS3 (Verschuere *et al.,* 1999, 2000; Marques *et al.,* 2005) for its positive effect on *Artemia* performance when fed sub-optimally and *Vibrio campbellii* strain LMG21363 (VC) for its pathogenic effect towards *Artemia* and shrimp (Marques *et al.,* 2005; Soto-Rodriguez *et al.,* 2003; Gomez-Gil *et al.,* 2004). The two bacterial strains were cultured and harvested according to procedures previously described by Marques *et al.* (2005). Pure cultures of the two bacterial strains were obtained from the Laboratory of Microbial Ecology and Technology, Gent University, and from the Laboratory of Microbiology, Gent University. The bacterial strains were stored at –80 °C and grown overnight at 28 °C on marine agar, containing Difco ™ marine broth 2216 (37.4 g/l, BD Biosciences) and agar bacteriological grade (20 g/l, ICN). For each bacterial srain a single colony was selected from the plate and incubated overnight at 28 °C in 50 ml Difco ™ marine broth 2216 on a shaker (150 rpm). Stationary-grown bacteria were harvested by centrifugation (15 min; ±2200 × *g*), the supernatant were discarded and the pellet resuspended in 20 ml filtered autoclaved sea water (FASW). Bacterial densities were determined by spectrophotometry (OD_{550}), assuming that an optical density of 1.000 corresponds to 1.2×10^9 cells/ml, according to McFarland standard (Biomerieux, Marcy l'Etoile, France).

At day 3, challenge tests were performed with live VC. For that purpose, in a laminar flow hood, the pathogen was provided to each replicate at a density of 5×10^6 cells/ml. Dead LVS3 was provided to *Artemia* using aliquots of autoclaved concentrated bacteria (autoclaving at 120°C for 20 min). After autoclaving, bacteria were plated to check if they were effectively killed by this method. For this purpose, 100µl of the culture medium were transferred to marine agar (MA; n=3), containing Difco™ marine broth 2216 (BD Biosciences, 3.74% w/v) and agar bacteriological grade (ICN, 2% w/v). Absence of bacterial growth was monitored after incubating plates for 5 days at 28°C. Autoclaving treatment was 100% effective, since no bacterial growth was observed on the MA after 5 days of incubation. Dead and live bacterial suspensions were stored at 4°C until the end of each experiment.

2.3. Yeast and bacterial ash-free content

To determine the yeast and bacterial ash free dry weight (AFDW), 50 ml of each culture sample were filtered on pre-dried filters (pore size 0.45 µm, two replicate per culture). Filters were subsequently dried at 60 °C for 24 h and weighed. Afterwards they were combusted at 600°C for 6 h to determine the ash content. The AFDW was calculated as the difference between dry weight and ash weight. The DW and AFDW of control (filter only, n=2) were subtracted from all samples. The AFDW of the yeast strains and the bacteria is presented in Table 3.2.

Table 3.2 Average ash free dry weight (AFDW) of 7 different null-mutants of yeast (isogenic strains derived from BY 4741) and the wild type strain (WT) harvested in the exponential and stationary growth phase, together with AFDW of dead LVS3 and live VC bacteria expressed in mg/10^9 cells. Values of AFDW are presented with the respective standard deviation (mean±S.D.). Values in the same column showing the same superscript letter are not significantly different (p_{Tukey}>0.05). *p*-values obtained for direct comparison of AFDW (mg/Falcon tube) of different yeast cell strains, harvested in exponential and stationary growth phase were included. Significant differences were obtained when $p<_{Tukey}0.05$.

Strains	AFDW (mg/10^9 cells)		AFDW (mg/Falcon tube)		*p*-value
	Exponential phase	Stationary phase	Exponential phase	Stationary phase	Exponential vs stationary phase AFDW (mg/Falcon tube)
WT	15.24±0.18^{f}	13.69±0.07^{d}	1.60±0.02^{e}	1.44±0.01^{de}	0.014
mnn9	54.67±1.66^{a}	36.40±7.23^{a}	5.74±0.17^{a}	3.82±0.75^{a}	0.161
mnn6	17.09±0.37^{e}	11.83±0.10^{de}	1.79±0.04^{d}	1.24±0.01^{e}	0.013
fks1	18.90±1.41^{d}	17.73±0.28^{c}	1. 98±0.13^{d}	1.86±0.0^{cd}	0.644
knr4	14.77±0.26^{f}	13.17±0.13^{d}	1.55±0.03^{e}	1.38±0.10^{de}	0.037
kre6	34.54±1.41^{b}	24.52±1.25^{b}	3.63±0.28^{b}	2.57±0.13^{b}	0.076
chs3	16.4±0.12^{e}	11.0±0.40^{e}	1.72±0.01^{d}	1.15±0.04^{e}	0.020
gas1	29.09±0.86^{c}	20.30±2.60^{bc}	3.05±0.09^{c}	2.13±0.28^{bc}	0.1
live LVS3	-	0.2186±0.02^{f}	-	0.023±0.01^{f}	-
dead LVS3	-	0.2725±0.02^{f}	-	0.029±0.01^{f}	-
live VC	-	0.1134±0.01^{f}	-	0.034±0.01^{f}	-

2.4. Artemia gnotobiotic culture

Experiments were performed with *Artemia franciscana* cysts, originating from Great Salt Lake, Utah-USA (EG ® type, INVE Aquaculture, Belgium). Bacteria –free cysts and nauplii were obtained using the procedure described by Marques *et al.* (2004 a). After hatching, 20 nauplii (Instar II) were picked and transferred to Falcon tubes containing 30 ml of FASW together with the amount of feed scheduled for day 1. Feeding rates were intended to provide *ad libitum* ratios but avoiding excessive feeding in order not to affect the water quality in the test tubes, except in experiments 4 and 5 (treatments 19-20) where nauplii were overfed (5.74 mg AFDW/FT) (Table 3.3, feeding regime: d) in order to verify the effect of overfeeding. Each treatment consisted of four Falcon tubes (replicates). Falcon tubes were placed on a rotating rod at 4 cycles per min, exposed to constant incandescent light (± 41μEm^{-2}) at 28°C. Tubes were being transferred to the laminar flow just once per day for feeding.

Table 3.3 Feeding regimes in the 3 experiments (Exp) performed. Daily and average total ash free dry weight (AFDW), expressed in μg/FT) of yeast cells and dead bacteria (LVS3) supplied to *Artemia* in experiment 3, 4 and 5. Challenge tests were performed with live *Vibrio campbellii* (VC) at a density of 5×10^6 cells/ml added at day 3 in experiment 4 and 5. Legend: a) dead LVS3+ yeast 5%; b) dead LVS3+ yeast 10%; c) the treatment dead LVS3 X; d) the treatment: dead LVS3 2X; X = the total amount of feed offered (2870 μg AFDW/FT); Y = yeast (wild type and isogenic yeast mutants added at 5% or 10%); DB = dead bacterium LVS3.

Feeding regime	Day1		Day2		Day3		Day4		Day5		Total AFDW offered (μg/FT)
	Y	DB	Y	DB	Y	DB	Y	DB	Y	DB	
Exp 3:	124	0	248	0	248	0	324	0	496	0	1440
Exp 4-5:											
a)	25	221	50	442	50	442	62	592	100	886	2870
b)	50	197	100	394	100	394	124	525	200	786	2870
c)	0	246	0	492	0	492	0	656	0	984	2870
d)	0	492	0	984	0	984	0	1312	0	1968	5740

2.5. Method used to verify axenity

Axenity of feed, decapsulated cysts and *Artemia* cultures was checked at the end of each experiment using a combination of plating (MA) and live counting (using tetrazolium salt MTT staining following the procedure described by Marques *et al.* (2004 a,b). In challenge treatments, the axenity of *Artemia* culture was always checked before challenge using the same methods. Contaminated culture tubes were not considered for further analysis and the treatment was repeated.

2.6. Experimental design

This study comprised 5 experiments and their experimental design was schematized in Fig. 3.1. In experiment 1, all live and axenic yeast strains (WT and 7 null mutants) were harvested in the exponential growth phase and used as feed for the *Artemia.*

In experiment 2, stationary-grown live and axenic yeast strains (the same strains as used in experiment 1) were used as feed for nauplii. In both experiments, a modified feeding schedule was adopted from Coutteau *et al.* (1990) and Marques *et al.* (2004 a,b). The feeding schedule resulted in an equal amount of yeast-cell particles per treatment being offered to *Artemia*. Both experiments were performed twice (A and B), to verify the reproducibility of the results.

In experiment 3, an equal amount of feed was provided to *Artemia* (Table 3.3). As the AFDW per cell of the yeast mutants is different (see Table 3.2), this resulted in different amount of yeast cells being offered. Each feed was tested in four replicates.

In experiment 4 & 5, all treatments were fed with an equal amount of yeast (in terms of AFDW). Yeast strains (in exponential and/or stationary growth phase) were provided daily in small but equal amounts, in combination with dead LVS3 (as a major part of the feed) to *Artemia* (Table 3.3- feeding regime for Exp. 4-5). As a control, *Artemia* was fed only dead LVS3 (Table 3.3- feeding regime: c). Challenge tests were performed with live VC at a density of 5×10^6 cells/ml added at day 3.

Fig. 3.1 Experimental design of the 5 experiments (Exp) performed. Legend: a)-g) correspond to the treatments performed; Y – yeast strains (wild type or isogenic yeast mutants). Yeast strains were added either at an equal amount of yeast cell particles (Exp 1 and 2) or an equal amount of feed (Exp3) or 5% or 10% (Exp 4 and 5); DB – dead bacterium LVS3; X - the total amount of dead LVS3 offered over the full experimental period (2870 µg AFDW/FT); P – pathogen (*Vibrio campbellii*).

		Day 1 Start		Day 2		Day 3		Day 4		Day 5		Day 6 Harvest
Exp 1-3	a)	Y	→	Y	→	Y	→	Y	→	Y	→	
Exp 4-5	b)	DB+Y	→	DB+Y	→	DB+Y	→	DB+Y	→	DB+Y	→	
	c)	DB+Y	→	DB+Y	→	DB+Y+P	→	DB+Y	→	DB+Y	→	
	d)	DB (X)	→	DB (X)	→	DB (X)	→	DB (X)	→	DB (X)	→	
	e)	DB (X)	→	DB (X)	→	DB (X)+P	→	DB (X)	→	DB (X)	→	
	f)	DB (2X)	→	DB (2X)	→	DB (2X)	→	DB (2X)	→	DB (2X)	→	
	g)	DB (2X)	→	DB (2X)	→	DB (2X)+P	→	DB (2X)	→	DB (2X)	→	

2.7. Survival and growth of Artemia

Survival and growth of *Artemia* nauplii were determined according to procedures described by Marques *et al.* (2004 a,b). At the end of experiment 1, 2 and 3 (day 6 after hatching) the number of swimming larvae was determined and survival percentage was calculated. Living larvae were fixed with Lugol's solution allowing to measure their individual length (growth calculation), using a dissecting microscope equipped with a drawing mirror, a digital plan measure and the software Artemia 1.0®(Marnix Van Domme). In order to integrate the results of survival and growth, the criterion "total length" was introduced, *i.e.* total millimeters of *Artemia* per Falcon tube or mm/FT = numbers of survivors × mean individual length.

In experiments 4 and 5 the survival percentage for each treatment was determined daily. For this purpose, the number of live *Artemia* was registered before feeding (or adding any bacteria) by exposing each transparent Falcon tube to an incandescent light without opening the tube to preserve the axenity.

Values of larval survival (percentage) were arcsin transformed, while values of individual length and total length were logarithmic or square root transformed to satisfy normal distribution and homocedastity requirements. Differences on survival, individual length and total length of *Artemia* fed with different feeds, were studied with analysis of variances (ANOVA) and multiple comparisons of Tukey's range, tested at 0.05 level of probability, using the software SPSS 11.5 for Windows.

3. Results

3.1. Artemia performance fed live yeast cells

Artemia nauplii were fed with 7 different isogenic mutant strains of baker's yeast (*Saccharomyces cerevisiae*) (Table 3.1) and compared with nauplii fed wild type yeast under gnotobiotic condition. In all cases equal amounts of yeast cells were offered. The results presented in Table 3.4 and 3.5 (results obtained in experiment 1 and 2) show that independently of the growth stage, the yeast genetic background has a big influence on *Artemia* performance. Compared with WT yeast, total biomass production of nauplii was significantly improved when the exp-grown isogenic yeast mutant strains were used as feed, due to both significant higher survival and/or individual length (Table 3.4). Among them, the mnn9 yeast strain supported the best nauplii performance.

Also the use of the mmn6 mutant resulted in a significantly improved total biomass production of *Artemia*. In this treatment a higher biomass production was obtained due to a considerable increase in survival. Using knr4 and fks1 (less β-1,3-glucans) and kre6 (less β-1,6-glucans) as feed resulted in less *Artemia* biomass production compared to the mnn9 yeast strain but significantly more *Artemia* biomass production compared to the WT-yeast. The chitin-defective yeast strain (chs3) supported a

small increase in *Artemia* biomass production compared to the WT-treatment, mainly due to better nauplii survival. Finally, using the gas1 mutant as food (this strain is lacking an important cell-wall protein involved in cross linking the major cell-wall components) resulted in better nauplii performance compared to WT yeast.

Higher total biomass (except for fks1 fed *Artemia*) production (compared to WT) in *Artemia* fed mutant stat-grown yeast cells were mainly due to higher nauplii survival rather than stronger individual growth. Only with stat-grown mnn9 cells higher survival and stronger individual growth contributed to more biomass production. When exp-yeast cells were fed to the nauplii, significant higher total *Artemia* biomass production (mostly due to higher nauplii survival) values were observed in all cases compared with stat-yeast cells possibly because the AFDW of yeast cells in the exponential growth phase was higher than in the stationary growth phase (Table 3.2) (although not significantly different in the mnn9, gas1 and fks1 yeast strains).

Table 3.4 Experiment 1: average survival (%), individual length (mm) and total length (mm per Falcon tube-FT) of *Artemia* nauplii fed live yeast cells (harvested in exponential growth phase) after 5 days: effect of growth stage and genetic background. Means were put together with the standard deviation (mean±S.D.). Each experiment was repeated twice A and B. Each feed was tested in four replicates. Values in the same column showing the same superscript letters are not significantly different (p_{Tukey}>0.05).

A				B		
Strains	Survival (%)	Individual length (mm)	Total length (mm/FT)	Surviva l (%)	Individual length (mm)	Total lenght (mm/FT)
WT	32±6^{c}	1.3±0.1^{f}	8.6±1.7^{d}	29±7^{c}	2.2±0.1^{cd}	12.5±3.3^{d}
mnn9	87±9^{a}	4.0±0.4^{a}	70.7±7.7^{a}	87±6^{a}	3.9±0.2^{a}	68.7±5.0^{a}
mnn6	64±7^{bc}	1.8±0.1^{de}	23.5±2.6^{c}	52±6^{bc}	1.8±0.2^{d}	19.0±2.3^{cd}
fks1	55±10^{b}	2.1±0.1^{c}	23.6±4.6^{c}	50±12^{bc}	2.2±0.3^{cd}	20.0±9.0^{cd}
gas1	61±5^{b}	3.2±0.2^{b}	39.0±3.0^{b}	64±5^{b}	3.3±0.1^{b}	42.0±3.0^{b}
knr4	62±6^{b}	2.0±0.2^{cd}	25.0±3.4^{c}	50±12^{bc}	2.3±0.1^{c}	23.0±5.6^{cd}
kre6	45±4^{bc}	2.0±0.1^{cd}	18±1.6^{c}	58±13^{b}	2.5±0.1^{c}	29.0±6.0^{c}
chs3	55±5^{b}	1.7±0.2^{e}	18.5±2.0^{c}	42±5^{bc}	2.0±0.2^{cd}	17.0±2.0^{cd}

Table 3.5 Experiment 2- average survival (%), individual length (mm) and total length (mm per Falcon tube-FT) of *Artemia* nauplii fed live yeast cells (harvested in the stationary growth phase) after 5 days: effect of growth stage and genetic background. means were put together with the standard deviation (mean±S.D.). Each experiment was repeated twice A and B. Each feed was tested in four replicates. Values in the same column showing the same superscript letters are not significantly different (p_{Tukey}>0.05).

A				B		
Strains	Survival (%)	Individual length (mm)	Total length (mm/FT)	Survival (%)	Individual length (mm)	Total lenght (mm/FT)
WT	15±7^{d}	1.75±0.2^{bcd}	5.2±2.5^{c}	25±4^{c}	1.7±0.2^{bc}	8.5±1.4^{c}
mnn9	68±5^{a}	3.3±0.2^{a}	45.6±3.2^{a}	71±4^{a}	2.8±0.1^{a}	40.0±2.7^{a}
mnn6	40±4^{bc}	1.8±0.1^{bc}	14.4±1.5^{b}	38±8^{bc}	1.3±0.1^{d}	9.8 ±1.9^{bc}
fks1	18±3^{d}	1.7±0.2^{bcd}	6.6±0.9^{c}	28±13^{bc}	1.6±0.2^{bc}	9.4±1.9^{bc}
gas1	35±4^{bc}	2.0±0.3^{b}	14.3±1.7^{b}	40±7^{b}	1.8±0.1^{b}	14.9±2.6^{b}
knr4	48±5^{b}	1.3±0.2^{d}	13.2±1.3^{b}	35±4^{bc}	1.5±0.1^{cd}	10.4±1.2^{bc}
kre6	40±13^{bc}	1.6±0.1^{cd}	12.6±4.0^{b}	32±15^{b}	1.6±0.2^{bcd}	12.2±2^{bc}
chs3	29±8^{cd}	1.7±0.2^{bcd}	9.7±2.9^{bc}	25±6^{bc}	1.9±0.1^{b}	9.3±2.2^{bc}

In the experiments described above equal amounts of yeast cell particles (Table 3.3 - feeding regime for Exp.3) were supplied as food, resulting, however, in different amounts of AFDW of food offered to *Artemia* (see Table 3.2). This could have been the reason for the considerable higher *Artemia* biomass production e.g. with mnn9 yeast. Hence, the feeding experiment was repeated, this time offering equal amounts of food expressed as AFDW. In all cases, feeding mutant-yeast cells resulted in better nauplii survival than feeding WT yeast. Also in this experiment, the mnn9-fed *Artemia* presented the highest biomass production (Table 3.6). *Artemia* biomass production was equal after feeding WT, fks1, kre6 and chs3 cells although the mutants (kre6 and knr4) display respectively a higher or equal AFDW as compared with WT yeast.

Table 3.6 Experiment 3: average survival (%), individual length (mm) and total length (in mm/ Falcon tube (FT)) of *Artemia* nauplii fed live yeast cells (harvested in the stationary growth phase) for 5 days: Effect of genetic background. All treatments were fed with an equal amount of feed corresponding to an AFDW (1.44±0.01mg/FT) (see Table 3.3- exp 3) (for instance fed with WT-YNB yeast cells). Means were put together with the standard deviation (mean±S.D.). Each feed was tested in four replicates. Values in the same column showing the same superscript letters are not significantly different (p_{Tukey}>0.05).

Strains	Survival (%)	Individual length (mm)	Total length (mm/FT)
WT	18 ± 6^{c}	1.74 ± 0.1^{c}	6.1 ± 2.2^{d}
mnn9	63 ± 6^{a}	2.44 ± 0.1^{a}	30.6 ± 3.2^{a}
mnn6	35 ± 9^{b}	1.8 ± 0.1^{c}	12.6 ± 3.3^{bc}
fks1	28 ± 7^{bc}	1.67 ± 0.1^{c}	9.2 ± 2.2^{cd}
gas1	38 ± 6^{b}	2.11 ± 0.1^{b}	15.8 ± 2.7^{b}
knr4	43 ± 6^{b}	1.53 ± 0.1^{d}	13.0 ± 2.0^{bc}
kre6	33 ± 7^{b}	1.33 ± 0.1^{e}	8.6 ± 1.7^{cd}
chs3	33 ± 6^{b}	1.81 ± 0.1^{c}	11.8 ± 2.3^{bcd}

3.2. Artemia performance fed bacteria (LVS3) and yeast and challeneged

The effect of feeding two different amounts of dead bacteria (autoclaved LVS3) to the nauplii (either challenged or not with a live pathogen) is presented in Table 3.7 (experiment 4). Unchallenged, there is no effect on survival in the two feeding regime. Yet, nauplii fed with dead LVS3 (5.74 mg AFDW/FT) (Table 3.3, feeding regime: d) yielded a significantly higher survival in the challenge test as compared to the nauplii fed with only half of this amount (Tables 3.7, lines 20 and 22). The results of supplying a low amount of WT or isogenic yeast mutants to the nauplii fed dead LVS3 are presented in Table 3.7 and 3.8. Most of the unchallenged nauplii fed solely with dead LVS3 or both dead LVS3 and yeast cells survived until day 6 (68 % or higher). In most treatments, challenged nauplii fed only with dead LVS3 or both dead LVS3 and yeast cells (WT, mnn6, fks1, knr4, kre6) died

before day 5. Yet, exceptions occurred when the nauplii were fed both dead LVS3 and mnn9, gas1 and chs3 (only exp-yeast) yeast strains. In challenge treatments, only mmn9-fed nauplii could survive until day 6 (Tables 3.7 and 3.8, line 4). However, the addition of 5% of the total AFDW in the form of mnn9 yeast cells was not sufficient to keep the nauplii alive until day 6 (Tables 3.7 and 3.8, line 6). In all yeast strains, the exp-yeasts provided a higher survival against the pathogen than stat-yeasts, but in most cases, this better survival lasted only for a short period (one day after challenge). The mnn9 cells supported a high survival until the end of the experiments against the VC pathogen when offered as exp-grown or stat-grown cells (Tables 3.7 and 3.8, line 4).

Table 3.7 Experiment 4: mean daily survival (%) of *Artemia* fed daily with dead LVS3 and yeast cell strains harvested in the exponential growth phase. The yeast cells constituted either 574±0.2 µg/ Falcon tube (FT) or 287±0.2 µg/FT of the total Ash Free Dry Weight (AFDW) supplied. The challenged test was performed with *Vibrio campbellii* (VC) added at day 3. Each experiment was repeated twice: A and B. Each feed was tested in four replicates. The total amount of feed offered is equal to 2870µg AFDW/FT. X- The total amount of dead LVS3 offered over the full experimental period (2870µg AFDW/FT). (Means were put together with the standard deviation (mean±SD.). Survival in the challenge test was compared directly to the survival of unchallenged *Artemia*. Values showing the same superscript letter are not significantly different ($p > 0.05$)

TN	A Survival(%)					B Survival(%)				
	Day2	Day3	Day4	Day5	Day6	Day2	Day3	Day4	Day5	Day6
1 dead LVS3+ WT 10%	93±5^{a}	86±5^{a}	81±5^{a}	74±4^{a}	68±5^{a}	91±5^{a}	88±5^{a}	83±5^{a}	73±5^{a}	66±5^{a}
2 dead LVS3+WT 10%+VC D3	91±5^{a}	88±5^{a}	56±5^{b}	0^{b}	0^{b}	88±5^{a}	86±5^{a}	55±4^{b}	0^{b}	0^{b}
3 dead LVS3+ mnn9 10%	98±5^{a}	94±5^{a}	90±4^{a}	86±5^{a}	82±5^{a}	96±5^{a}	93±5^{a}	91±5^{a}	84±5^{a}	80±4^{a}
4 dead LVS3+ mnn9 10%+VC D3	96±5^{a}	93±5^{a}	86±5^{a}	80±4^{a}	78±5^{a}	98±5^{a}	93±5^{a}	88±5^{a}	81±5^{a}	76±5^{a}
5 dead LVS3+mnn9 5%	98±5^{a}	93±5^{a}	86±5^{a}	80±4^{a}	75±4^{a}	98±5^{a}	93±5^{a}	88±5^{a}	84±5^{a}	76±5^{a}
6 dead LVS3+mnn9 5%+VC D3	99±5^{a}	89±5^{a}	84±5^{a}	31±5^{b}	0^{b}	98±5^{a}	91±5^{a}	81±5^{a}	35±4^{b}	0^{b}
7 dead LVS3+mnn6 10%	93±5^{a}	89±5^{a}	83±5^{a}	79±5^{a}	73±5^{a}	91±5^{a}	89±5^{a}	81±5^{a}	78±5^{a}	70±4^{a}
8 dead LVS3+mnn6 10%+VC D3	91±5^{a}	88±5^{a}	66±5^{b}	0^{b}	0^{b}	96±5^{a}	86±5^{a}	70±4^{b}	0^{b}	0^{b}
9 dead LVS3+Hxs1 10%	93±5^{a}	86±5^{a}	83±5^{a}	79±5^{a}	70±4^{a}	93±5^{a}	86±5^{a}	81±5^{a}	78±5^{a}	73±5^{a}
10 dead LVS3+Hxs1 10%+VC D3	93±5^{a}	88±5^{a}	64±5^{b}	0^{b}	0^{b}	91±5^{a}	88±5^{a}	66±5^{b}	0^{b}	0^{b}
11 dead LVS3+kre4 10%	94±5^{a}	88±5^{a}	79±5^{a}	76±4^{a}	73±5^{a}	93±5^{a}	86±5^{a}	81±5^{a}	75±4^{a}	69±5^{a}
12 dead LVS3+kre4 10%+VC D3	93±5^{a}	86±5^{a}	63±5^{b}	0^{b}	0^{b}	93±5^{a}	88±5^{a}	66±5^{b}	0^{b}	0^{b}
13 dead LVS3+kre6 10%	94±5^{a}	88±5^{a}	80±4^{a}	75±4^{a}	71±5^{a}	93±5^{a}	88±5^{a}	83±5^{a}	78±5^{a}	70±4^{a}
14 dead LVS3+kre6 10%+VC D3	94±5^{a}	88±5^{a}	60±4^{b}	0^{b}	0^{b}	93±5^{a}	86±5^{a}	65±4^{b}	0^{b}	0^{b}
15 dead LVS3+gas1 10%	93±5^{a}	88±5^{a}	84±5^{a}	81±5^{a}	73±5^{a}	94±5^{a}	89±5^{a}	86±5^{a}	78±5^{a}	71±5^{a}
16 dead LVS3+gas1 10%+VC D3	94±5^{a}	86±5^{a}	50±4^{b}	36±4^{b}	0^{b}	93±5^{a}	88±5^{a}	52±6^{b}	33±6^{b}	0^{b}
17 dead LVS3+ths3 10%	94±5^{a}	86±5^{a}	79±5^{a}	70±5^{a}	73±5^{a}	96±5^{a}	88±5^{a}	81±5^{a}	75±4^{a}	74±5^{a}
18 dead LVS3+ths3 10%+VC D3	94±5^{a}	88±5^{a}	75±6^{a}	34±5^{b}	0^{b}	98±5^{a}	86±5^{a}	61±5^{a}	20±5^{b}	0^{b}
19 dead LVS3(2X)	98±5^{a}	93±5^{a}	84±5^{a}	78±5^{a}	73±5^{a}	98±5^{a}	89±5^{a}	85±4^{a}	78±5^{a}	74±5^{a}
20 dead LVS3(2X)+VC D3	98±5^{a}	86±5^{a}	77±5^{a}	53±5^{b}	44±5^{b}	99±5^{a}	86±5^{a}	70±5^{a}	65±4^{b}	51±5^{b}
21 dead LVS3 (X)	98±5^{a}	90±5^{a}	83±5^{a}	76±5^{a}	69±5^{a}	96±5^{a}	91±5^{a}	83±5^{a}	75±4^{a}	68±5^{a}
22 dead LVS3 (X)+VC D3	96±5^{a}	89±5^{a}	56±5^{b}	0^{b}	0^{b}	98±5^{a}	93±5^{a}	60±4^{b}	0^{b}	0^{b}

Table 3.8 Experiment 4: mean daily survival (%) of *Artemia* fed daily with dead LVS3 and yeast cell strains harvested in the stationary growth phase. The yeast cells constituted either 574±0.2 µg/ Falcon tube (FT) or 287±0.2 µg/FT of the total Ash Free Dry Weight (AFDW) supplied. The challenged test was performed with *Vibrio campbellii* (VC) added at day 3. Each experiment was repeated twice: A and B. Each feed was tested in four replicates. The total amount of feed offered is equal to 2870µg AFDW/FT. X- The total amount of dead LVS3 offered over the full experimental period (2870µg AFDW/FT). (Means were put together with the standard deviation (mean±SD.). Survival in the challenge test was compared directly to the survival of unchallenged *Artemia*. Values showing the same superscript letter are not significantly different ($p>0.05$)

TN	A Survival (%)					B Survival (%)				
	Day2	Day3	Day4	Day 5	Day 6	Day2	Day3	Day4	Day5	Day6
1 dead LVS3+ WT 10%	93±3^a	88±3^a	79±5^a	75±3^a	65±4^a	91±3^a	88±3^a	76±3^a	73±3^a	6±5^a
2 dead LVS3+WT 10%+VC D3	93±3^a	89±3^a	46±5^b	0^b	0^b	94±3^a	89±3^a	45±6^b	0^b	0^b
3 dead LVS3+ mnn9 10%	99±3^a	96±3^a	91±3^a	86±3^a	83±3^a	98±3^a	94±3^a	89±5^a	86±3^a	80±4^a
4 dead LVS3+ mnn9 10%+VC D3	98±3^a	95±3^a	86±3^a	81±3^a	79±3^a	98±3^a	93±3^a	86±3^a	83±3^a	76±3^a
5 dead LVS3+mnn9 5%	94±3^a	89±3^a	81±5^a	75±4^a	73±3^a	98±3^a	91±3^a	80±6^a	75±4^a	71±3^a
6 dead LVS3+mnn9 5%+VC D3	91±3^a	88±3^a	71±5^a	23±3^b	0^b	96±3^a	90±4^a	74±3^b	25±4^b	0^b
7 dead LVS3+mnn6 10%	99±3^a	91±3^a	84±3^a	78±3^a	70±4^a	99±3^a	93±3^a	83±3^a	76±5^a	69±5^a
8 dead LVS3+mnn6 10%+VC D3	98±3^a	90±4^a	43±3^b	0^b	0^b	99±3^a	91±3^a	45±4^b	0^b	0^b
9 dead LVS3+fks1 10%	98±3^a	91±3^a	81±3^a	76±3^a	68±3^a	98±3^a	91±3^a	84±3^a	75±4^a	69±3^a
10 dead LVS3+fks1 10%+VC D3	96±3^a	90±4^a	41±5^a	0^b	0^b	96±3^a	90±4^a	39±5^b	0^b	0^b
11 dead LVS3+knr4 10%	95±3^a	89±3^a	85±4^a	78±3^a	71±5^a	96±3^a	91±3^a	84±3^a	76±5^a	74±3^a
12 dead LVS3+knr4 10%+VC D3	99±3^a	88±3^a	46±5^b	0^b	0^b	98±3^a	93±3^a	50±4^b	0^b	0^b
13 dead LVS3+kre6 10%	98±3^a	84±3^a	79±3^a	78±3^a	69±5^a	96±3^a	88±3^a	78±3^a	79±3^a	71±5^a
14 dead LVS3+kre6 10%+VC D3	96±3^a	86±3^a	41±5^b	0^b	0^b	98±3^a	86±3^a	38±3^b	0^b	0^b
15 dead LVS3+gas1 10%	91±3^a	88±3^a	84±3^a	73±3^a	69±3^a	93±3^a	89±3^a	83±3^a	74±5^a	71±3^a
16 dead LVS3+gas1 10%+VC D3	90±3^a	89±3^a	43±3^b	25±4^b	0^b	93±3^a	86±3^a	46±3^b	28±3^b	0^b
17 dead LVS3+chs3 10%	98±3^a	88±3^a	83±3^a	75±4^a	68±3^a	96±3^a	89±5^a	84±3^a	78±3^a	70±4^a
18 dead LVS3+chs3 10%+VC D3	98±3^a	89±3^a	68±3^b	0^b	0^b	98±3^a	88±3^a	66±5^b	0^b	0^b

Table 3.9 Summary table of results obtained in the challenged and unchallenged experiments: "A-B" means respectively statistically different (p<0.05) strong and moderate positive effect of yeast feed on *Artemia* performance in comparison to the wild-type yeast strain. C means no statistically effect of feed on *Artemia* performance in comparison to the wild-type yeast strain. "+" means protection (no significant difference in survival rate between challenged and unchallenged treatments) provided by small amounts of yeast feed (5 or 10%) on *Artemia* performance when fed with dead LVS3 and challenged with *Vibrio campbellii* . "-" means no protection (significant difference in survival rate between challenged and unchallenged treatments). A circle around the "+" means that the feed was only protecting partially against the pathogen. D4, D5 and D6 correspond respectively to day 4, 5 and 6.

		Unchallenged experiments (Exp1-3)			Challenged experiments (Exp 4 -5)		
Yeast strains	Phenotype	Survival (D6) (%)	IL (mm)	TBP (mm/FT)	survival (D4) (%)	survival (D5) (%)	survival (D6) (%)
WT	control yeast	C	C	C	+	-	-
mnn9	less mannan, higher chitin, higher β- glucans	A	A	A	+	+	+
mnn6	less phosphomannan	B	C	C	+	-	-
fks1	less β1,3 glucans, higher chitin	B	C	C	+	-	-
knr4	less β1,3 glucans, higher chitin	B	C	C	+	-	-
kre6	less β1,6 glucans, higher chitin	B	C	C	+	-	-
chs3	less chitin	B	C	C	+	⊕	-
gas1	less integration of yeast cell adhesion proteins into the cell wall less β1,3 glucans, higher chitin	B	B	B	+	⊕	-

4. Discussion

Marques *et al.* (2004 a,b) have shown that yeast digestion by *Artemia* can be significantly improved by manipulating the genetic characteristics of the yeast, the growth stage and the medium used. This study confirms that the genetic background of the yeast strain used, has a strong influence on the *Artemia* performance (Experiment 1, 2 and 3).

In this study, a yeast strain containing low concentrations of mannoproteins in the cell wall, such as the mnn9 mutant, always supported a high *Artemia* biomass production (*i.e.* best nauplii growth as well as highest survival rate). According to Coutteau *et al.* (1990), β-glucanase activity is detected in the digestive tract of *Artemia* but no mannase activity, making the external mannoprotein layer of the yeast cell wall probably the major barrier for the digestion of the yeast cell by the *Artemia* nauplii. Therefore, it is likely that the digestive enzymes of *Artemia* (such as β-glucanase) can easily enter and provide suitable digestion of yeast cells with reduced mannoprotein content.

An improved *Artemia* performance was also obtained with yeast mutants with reduced β-glucans (fks1, knr4, kre6 and gas1) and chitin (chs3) levels as compared to the nauplii fed WT-yeast cells (especially with exp-grown yeast cells as food). According to Aguilar-Uscanga and Francois (2003), β-1,3 glucans and especially β-1,6 glucans provide anchorage to most cell-wall mannoproteins and are also covalently linked with chitin, contributing to the modular structure of the cell wall. β-1,3 glucans also contribute to the rigidity and integrity of the cell wall, and determine the cell shape (Martine-Yken *et al.,* 2002). As a consequence, a lack of β- glucans in the yeast cell wall might result in less covalent linkage between the three cell-wall compounds, resulting in a more permeable and digestible cell wall in comparison to the WT strain. Although in a WT yeast strain chitin concentration makes up only 1-2 % of the cell-wall dry mass (Magnelli *et al.,* 2002), it plays a key role in yeast cell growth and division and is attached covalently to β-1,3-glucans, β-1,6-glucans and mannoproteins (Cabib *et al.,* 2001). Therefore, the better results obtained with nauplii fed chitin defective yeast could also be due to an enhanced digestibility of chs3-cells by *Artemia*, caused by reduced linkage between the three cell wall components.

In the gas1 yeast mutant, the production of the glycosylphosphatidylinositol (GPI) anchored protein is inhibited resulting in a non-proper fiber assembly of the cell wall (defective architecture) as well as in reduced β-glucans (Ram *et al.,* 1998; Lipke and Ovalle, 1998). This apparently results in an eventually increased digestibility of gas1 cells by *Artemia.* Compared to WT-fed nauplii, yeast strains with reduced phosphomannan levels in the cell wall (mnn6) always gave higher *Artemia* performance mainly due to a higher nauplii survival. This fact could be due to interference of phosphomannans in phosphodiester cross-linking of mannoproteins to β-glucans (Jigami and Odani, 1999). In the experiments shown in Table 3.4 and 3.5, equal amounts of yeast cell particles (feeding schedule of exp. 1 and 2) are offered. This results in different amounts of AFDW being supplied (Table 3.2). We therefore, in a further experiment, supplied exactly equal amount of AFDW (see Table 3.3- feeding regime for exp. 3) of the different yeast cells to *Artemia* (Table 3.6). Consequently, in these experiments, the feed particle concentration, just after the feeding, was different with the various mutants. Also in this case mnn9-fed *Artemia* outperformed WT-fed *Artemia* (both in survival and individual growth). With the other mutants, *Artemia* biomass production improved mainly through higher survival. With two mutants, namely fks1 and chs3, *Artemia* biomass production was equal as in the experiment where WT-cells were offered.

In the present study the *Artemia* nauplii displayed a higher performance when fed an exp-grown cells as compared stat-grown yeast strains. According to Klis *et al.* (2002), yeast cells entering the stationary phase of growth will form different cell walls, i.e. thicker, more resistant to enzymatic breakdown and less permeable to macromolecules. The level of mannosyl phosphorylation of cell-wall proteins increases in the late-exponential and stationary phase of growth (Odani *et al.,* 1997). In addition more extensive cross-linking (through disulfide bridges) between the polysaccharide components of the cell wall (mannoproteins, glucans and chitin) is taking place in the stationary phase (Cabib *et al.,* 2001; Deutch and Parry, 1974; De Nobel *et al.,* 2004). In conclusion, it seems that the density of covalent linkage between the three cell-wall compounds of the yeast cell plays an important role in their digestibility by *Artemia*. In addition to that, high amounts of cell wall chitin and glucans in

combination with low amounts of mannoproteins favour *Artemia* biomass production under gnotobiotic condition (Marques *et al.,* 2004 a,b).

According to Raa (2000) improvements in the health status of aquatic organisms can be achieved by balancing the diet with regards to nutritional factors. This phenomenon is identified as nutritional immunology, since some nutritional factors are so closely linked with biochemical processes of the immune system that significant health benefits can be obtained by adjusting the concentration of such factors. Inadequate food or imbalances in the nutrient composition of the diet will affect growth and general performance of an animal, most likely, also the biochemical process of the immune system (Raa, 2000). In this study nauplii fed with dead LVS3 (5.74 mg AFDW/FT) (Table 3.4- feeding regime: d), presented significantly higher survival after challenge in comparison to nauplii fed solely with half of this amount (Tables 3.7). This experiment clearly illustrated that the outcome of the challenge with *Vibrio campbellii* under gnotobiotic condition is very much dependent on the overall condition of the nauplii. These results are also consistent with the perception that *Vibrio spp* are opportunistic pathogens. Therefore in all challenge experiments, in which the effect of yeast mutants were tested in small quantities, the total AFDW supplied was kept constant. The mnn9-fed *Artemia* could resist detrimental effect of pathogenic VC until the end of experiments as previously reported by Marques *et al.* (2005) (Tables 3.7 and 3.8). Nevertheless, the addition of 5% of mnn9 yeast was not able to protect nauplii against VC until day 6. The mnn9 yeast has a null mutation resulting in phenotypically increased amounts of cell wall bound chitin and glucans in combination with reduced amount of mannose linked to mannoproteins, and probably a reduced density of covalent linkages between these three yeast cell wall constituents and/or nature of the covalent bonds, in comparison to the WT strain (Magnelli *et al.,* 2002; Aguilar-Uscanga and François, 2003). The protection provided by the mnn9 yeast could be the effect of general improvements in *Artemia* health condition due to extra (or better quality) nutrients available in this yeast or due to a stimulation of a non-specific immune response by some compounds, such as β-glucans or chitin that are present in the yeast cell wall. Vismara *et al*. (2004) considerably increased *Artemia* resistance to stress conditions, such as poor

growth medium quality and daily handling, by administering daily nauplii with a mutant of *Euglena gracilis* presenting high amount of β-glucans (and thus enabling its response against disease). In contrast to mnn9 yeast, which has both strong nutritional and/or possible immunologic characteristics, gas1 cells have good nutritional effects, and protect nauplii temporarily against the pathogen in the challenge test. Furthermore, weak or no nutritional and protection effects were observed with fks1, knr4, mnn6 and kre6 yeast cells. Yet, interestingly, temporary protection against VC was obtained by adding chs3 in the diet, while this mutant has hardly any effect on individual growth (Table 3.7). Using the described set of yeast mutants, a full or partial protection against VC can be associated with increased glucan and chitin in the cell wall (*e.g.* mnn9 but also gas1) and reduced chitin and increased glucan in the cell wall (chs3). This seems to suggest that chitin as such is not involved in the protection against VC. Rather the results indicate that glucan as such is the potential active compound.

β-glucans have been identified as specific immunostimulants activating the aquatic organisms immune system and protecting them from adverse conditions (Anderson, 1992). For example, yeast *Saccharomyces cerevisiae* has been found to be a good enhancer of the trout immune system (Siwicki *et al.,* 1994). Patra and Mohamed (2003) showed that *Artemia* supplemented with *Saccharomyces boulardi* were protected against *Vibrio harveyi*. The results showed that chitin-enrichment in the fks1 strain may be responsible for increasing the innate immune responses resulting in beneficial effects on fish performance. The later findings are not supported by our results.

In conclusion, the mnn9 yeast strain, even in small quantities, can protect *Artemia* nauplii against pathogenic bacteria, suggesting that this yeast strain is possibly stimulating the innate immune response. It seems probably that mnn9 cells protect nauplii either through their higher concentration of β-glucans in the cell wall and/or the higher availability of β-glucans to nauplii. However, an overall nutritional stimulation by mnn9 with positive effect on the immunological status cannot be excluded. Using chs3 strain (in comparison to WT) as feed has very little extra effect on the growth and survival. Yet, this feed can temporarily protect *Artemia* against VC.

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CHAPTER IV

Anti-infectious potential of beta-mercaptoethanol treated baker's yeast in gnotobiotic *Artemia* challenge test

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Submitted

Anti-infectious potential of beta-mercaptoethanol treated baker's yeast in gnotobiotic *Artemia* challenge test

Abstract

A selection of isogenic yeast strains (with deletion for genes involved in cell-wall synthesis) was treated with 2-mercapto-ethanol (2ME) and then used to evaluate their nutritional and anti-infectious characteristics for gnotobiotically-grown *Artemia.* In the first set of experiments the effect of the chemical treatment on the yeast nutritional value was studied. In most cases, 2ME-treated yeast cells were better feed for *Artemia* than the untreated cells. The better performance of *Artemia* fed 2ME-treated cells was due either to an increased survival (with WT, knr4 and chs3) or a better individual growth (fks1 and kre6) or due to both (mnn6). The second set of experiments, investigated the use of a small quantity (10% of the total feed supplied) of 2ME-treated yeast cells in *Vibrio campbellii* (VC) challenged *Artemia*. The 2ME-treated gas1, kre6 and chs3 strains improved *Artemia* resistance against VC. However, the enhanced resistance with these strains is not concomitant with a better individual growth (e.g. as observed with mnn9-fed *Artemia),* suggesting non-interference of general nutritional effects for these three 2ME-treated strains. It is postulated that this simple chemical treatment in these strains could increase the bio-availability of protective compounds (such as β-glucans) to *Artemia,* stimulating its immune system.

1. Introduction

According to Raa (2000), invertebrates are apparently entirely dependent on non-specific immune mechanisms to cope with infections, as they lack the specific immunological "memory" that is found in fish and warm-blooded animals. As a result, it does not seem to make sense to vaccinate them against specific diseases. Nowadays, the use of preventive and environment-friendly approaches such as probiotics, immunostimulantns, antibacterial peptides and quorum sensing systems is becoming

increasingly important in aquaculture (Bachére *et al.,* 2003; Sakai, 1999; Verschuere *et al.,* 2000; Defoirdt *et al.,* 2005). However, the application of such technologies must be based on thorough understanding of the mechanisms involved and the putative consequences. An essential part of that understanding can be provided by studies looking in detail at host-microbial interactions. A key experimental approach to study these interactions is to define first the functioning of the host in the absence of bacteria and then to evaluate the effect of adding a single or defined population of microbes, or certain compounds (*i.e.,* under axenic or of certain gnotobiotic conditions) (Teunissen *et al.,* 1998; Anderson, 1992; Sung *et al.,* 1996). Marques *et al*. (2004a) have recently developed and validated the usefulness of an *Artemia* gnotobiotic test system allowing studying the effect of food composition as well as the host-microbial interaction on survival and growth of *Artemia* in the presence or absence of a pathogen.

Saccharomyces cerevisiae, which has been found to be a good immune enhancer in some aquatic organism (Siwicki *et al.,* 1994; Ortuño *et al.,* 2002; Li *et al.,* 2003, 2004) is an excellent source of β-glucans and chitin. These compounds together with mannoproteins constitute the major compounds of the yeast cell-wall (Magnelli *et al.,* 2002). In fact, the four major components of the yeast cell wall are β-1,3- glucan (50% of cell wall dry weight-DW) and chitin (1–2% of cell wall DW), mostly presented in the inner layer and β-1,6-glucan (8% of cell wall DW) and mannan (40–50% of cell wall DW), mostly present in the outer layer (Marques *et al.,* 2004a; Dallies *et al.,* 1998; Klis *et al.,* 2002; Keith *et al.,* 1992). According to Jigami and Odani (1999) mannoproteins located in the outer layer of the yeast cell wall determine the wall's porosity and thereby regulate leakage of proteins from the periplasmic space and entrance of macromolecules from the environment.

As reported by Couteau et *al.* (1990), β-glucanase activity is detected in the digestive tract of *Artemia* but no mannase activity is found, making the external mannoprotein layer of the yeast cell wall probably the major barrier to yeast digestion by *Artemia*. Several methods have been developed to improve the digestibility of Single Cell Proteins (SCP) such as mechanical distruption, autolysis and enzymatic treatment (Kihlberg, 1972; Hendenskog and Morgen, 1973). However, as a result of these

drastic treatments soluble cytoplasmatic contents in the yeast cells are exposed to the environment. As a consequence, yeast nutrients are lost to filter feeders and, moreover, culture conditions deteriorate due to reduced water quality. Coutteau *et al.,* (1990) proposed a chemical treatment, which makes the yeast digestible while maintaining the cell integrity. In a previous study (Soltanian *et al.,* 2007) the effect of isogenic yeast deletion mutants (8 strains), carrying a null mutation in a gene involved in cell wall synthesis, was evaluated in a gnotobiotic *Artemia* test system. The present study aims at investigating the influence of 2-beta-mercaptoethanol (2ME) treated yeast cells on *Artemia* performance in a gnotobiotic *Artemia* test system. Firstly, *Artemia* performance was examined with the yeast strains affected in their cell wall synthesis, (harvested in stationary growth phase) either treated or untreated with the reducing agent 2ME. In a second stage, these feed sources were tested in combination with a *Vibrio campbellii* challenge.

2. Methodology

2.1. Axenic yeast culture and preparation

To verify the digestibility of live baker's yeast (*S. cerevisiae*) by *Artemia,* 7 different null-mutants of yeast (isogenic deletion strains derived from baker's yeast strain BY 4741) and the wild type strain (WT) (genotype described in Table 4.1) were fed to *Artemia*. All strains were provided by EUROSCARF (University of Frankfurt, Germany).

Table 4.1 **Genotype of all yeast strains used as feed for Artemia and description of each gene mutation in the development of cell wall components.**

Strains	Genotype	Phenotype (cell wall changes)	Reference
WT	BY 4741*; Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0	control yeast	Dallies et al.(1998); Klis et al.(2002); Magnelli et al.(2002); Marques et al.(2004)
mnn9	BY4741; *Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0;YPL050c::kanMX4	less mannan, higher chitin, higher β- glucans	Klis et al.(2002); Klis et al.(2002); Magnelli et al.(2002); Marques et al.(2004)
mnn6	BY4741; *Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0;YPL053c::kanMX4	less phosphomannan	Karson and Ballou (1978); Wang et al (1997) Jigami and Odani (1999)
fks1	BY4741; *Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0;YLR342w::kanMX4	less β1,3-glucans, higher chitin	Dallies et al.(1998); Magnelli et al.(2002); Martin-Yken et al.(2002); Pagé et al.(2003) Aguillar-Uscana and Francois (2003)
knr4	BY4741; *Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0;YGR229c::kanMX4	less β1,3-glucans, higher chitin	Dallies et al.(1998); Magnelli et al.(2002); Martin-Yken et al.(2002); Pagé et al.(2003); Aguillar-Uscana and Francois (2003)
kre6	BY4741; *Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0;YPR159w::kanMX4	less β1,6-glucans, higher chitin	Magnelli et al.(2002); Francois(2003) ; Martin-Yken et al.(2002); Pagé et al.(2003)
chs3	BY4741; *Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0;YBR023c::kanMX4	less chitin	Valdivieso et al.(2000); Cabib et al.(2001); Klis et al.(2002); Magnelli et al.(2002);
gas1	BY4741; *Mat a; his 3 Δ1; leu 2Δ0;* met 15Δ0; ura3 Δ0;YMR307w::kanMX4	less integration of yeast cell adhesion proteins into the cell wall less β1,3- glucans, higher chitin	De Nobel et al.(1994); Popolo et al.(1997) Lipke and Ovalle (1998); Magnelli et al.(2002)

Yeasts cells were grown according to the procedures described by Marques *et al.* (2004a), using minimal Yeast Nitrogen Base culture medium (YNB). Yeasts were harvested by centrifugation (± *800* × *g* for 10min) in the stationary growth phase (after 3 days;"stat.yeast"). Chemical (2ME) treatment was applied on yeast cells according to the procedure described by Coutteau *et al.,* (1990). First, yeast cells were suspended at a concentration of 200 mg wet weight/mL in a sterilized medium containing Na_2EDTA (0.05 M) and Tris-buffer (0.2 M; pH 8). After addition of (2ME) (2% volume/volume) the yeasts were incubated for 30 minutes at 30°C. Pre-treated yeasts were collected and washed with protoplasting medium comprising a phosphate-citrate buffer (KH_2PO_4 0.08 M; Na_3citrate 0.016 M; pH 5.8) and KCl (0.6 M). Finally, yeast cells were washed three times with filtered (0.2µ) seawater. Yeast cell concentrations were determined with a Bürker haemocytometer. Yeast suspensions were stored at 4 °C until the end of each experiment (maximum storage of one week).

2.2. Bacterial strains and growth conditions

Two bacterial strains were used, *i.e. Aeromonas hydrophila* strain LVS3 (Verschuere *et al.,* 1999, 2000; Marques *et al.,* 2005) for its positive effect on *Artemia* performance when fed at sub-optimal concentration and *Vibrio campbellii* strain LMG21363 (VC) for its pathogenic effect towards *Artemia* and shrimp (Marques *et al.,* 2005; Soto-Rodriguez *et al.,* 2003; Gomez-Gil *et al.,* 2004). The two bacterial strains were cultured and harvested according to Marques *et al.* (2004a). Bacteria were resuspended in filtered and autoclaved seawater (FASW) and their densities determined by spectrophotometry (OD_{550}), assuming that an optical density of 1.000 corresponds to 1.2×10^9 cells/ml, according to the McFarland standard (Biomerieux, Marcy l'Etoile, France). At day 3, challenge tests were performed with live VC according as described by Soltanian *et al.* (2007).

2.4. Artemia gnotobiotic culture

Experiments were performed with *Artemia franciscana* cysts, originating from Great Salt Lake, Utah-USA (EG ® type, INVE Aquaculture, Belgium). Bacteria–free cysts and nauplii were obtained according to Sorgeloos *et al.* (1986). After hatching, 20 nauplii (Instar II) were picked and transferred to Falcon tubes containing 30 ml of FASW together with the amount of feed scheduled for day 1. Feeding rates were intended to provide *ad libitum* ratios but avoiding excessive feeding in order not to affect the water quality in the test tubes. Each treatment consisted of four Falcon tubes (replicates). Falcon tubes were placed on a rotating rod at 4 cycles per min, exposed to constant incandescent light ($\pm$ 41µEm^{-2}) at 28°C. Tubes were being transferred to the laminar flow just once per day for feeding.

2.5. Method used to verify axenity

Axenity of feed, decapsulated cysts and *Artemia* cultures was checked at the end of each experiment using a combination of plating (Marine Agar) and live counting (Marques *et al.* (2004a) (using tetrazolium salt MTT (-3-(4,5-dimethylthazol-2, 5-diphenyl tetrazolium bromide; Sigma, 0.5 % w/v). In challenge treatments, the axenity of *Artemia* culture was always checked before challenge using the same methods. Contaminated culture tubes were not considered for further analysis and the treatment was repeated.

2.6. Experimental design

This study comprises 3 experiments and their experimental design is schematised in Fig. 4.1. In experiment 1, stationary-grown live and axenic yeast strains (WT and 7 null mutants) both treated or untreated with 2ME were used as feed for nauplii. In this experiment, a modified feeding schedule was adopted from Coutteau *et al.* (1990) and Marques *et al.* (2004a). This experiment was performed twice (A and B), to verify the reproducibility of the results.

	Day 1 Start	Day 2	Day 3	Day 4	Day 5	Day 6 Harvest
Exp 1 a)	Y →	Y →	Y →	Y →	Y →	
b)	Y-T →	Y-T →	Y-T →	Y-T →	Y-T →	
Exp 2-3 c)	DB+Y →	DB+Y →	DB+Y →	DB+Y →	DB+Y →	
d)	DB+Y →	DB+Y →	DB+Y+P →	DB+Y →	DB+Y →	
e)	DB+Y-T →	DB+Y-T →	DB+Y-T →	DB+Y-T →	B+Y-T →	
f)	DB+Y-T →	DB+Y-T →	DB+Y-T+P →	DB+Y-T →	B+Y-T →	
g)	DB →	DB →	DB →	DB →	DB →	
h)	DB →	DB →	DB+P →	DB →	DB →	

Fig. 4.1 Experimental design of the 3 experiments (Exp) performed. Legend: a)-h) correspond to the treatments performed; Y: Untreated yeast strains (wild type or isogenic yeast mutants). Y-T: Yeast strains (wild type or isogenic yeast mutants) treated with 2ME. Yeast strains were added either at an equal amount of yeast cell particles (Exp 1) or 10% of the total AFDW supplied (287 µg/FT) (Exp 2 and 3); DB – dead bacterium LVS3 which was offered over the full experimental period (2870 µg AFDW/tube); P – pathogen (*Vibrio vampbellii)* added on day 3.

In experiment 2 and 3, all treatments were fed with an equal amount of feed on a dry weight basis. Yeast strains were provided daily in small but equal amounts (only 10% of total ash free dry weight (AFDW) supplied which is equal to 287 µg/tube) in combination with dead LVS3 (as a major part of the feed) to *Artemia* (Table 4.2- feeding regime for Exp. 2 and 3). The total amount of dead LVS3 provided to *Artemia* was approximately 10.5×10^9 cell/tube that is equal to 2870 µg AFDW/tube (distributed in 5 daily feeding portions based on the fraction 9:17:17:23:34 and added daily to *Artemia* over the experimental period). The total amount of AFDW of yeast and bacteria added to *Artemia* in experiment 2 and 3 is presented in Table 4.2. As a control, *Artemia* was fed only dead LVS3 (Table

4.2- feeding regime: b). Challenge tests were performed with live VC at a density of 5×10^6 cells/ml added at day 3.

Table 4.2 Feeding regimes in the 2 experiments (Exp) performed. Daily and average total ash free dry weight (AFDW), expressed in µg/FT of yeast cells and dead bacteria (LVS3) supplied to *Artemia* in experiment 1 and 2. Challenge tests were performed with live *Vibrio campbellii* (VC) at a density of 5×10^6 cells/ml added at day 3 in experiment 2 and 3. Legend: a) dead LVS3+ yeast 10%; b) the control treatment: dead LVS3; Y = yeast (wild type and isogenic yeast mutants treated and/or untreated added 10%); DB = dead bacterium LVS3.

Feeding regime	Day1		Day2		Day3		Day4		Day5		Total AFDW offered (µg/FT)
	Y	DB	Y	DB	Y	DB	Y	DB	Y	DB	
Exp 2-3:											
a)	25	221	50	442	50	442	62	592	100	886	2870
b)	0	246	0	492	0	492	0	656	0	984	2870

2.7. Survival and growth of Artemia

Survival and growth of *Artemia* nauplii were determined according to procedures described by Marques *et al.* (2004a). At the end of experiment 1 and 2 (day 6 after hatching) the number of swimming larvae was determined and survival percentage was calculated. Living larvae were fixed with Lugol's solution allowing to measure their individual length (growth calculation), using a dissecting microscope equipped with a drawing mirror, a digital plan measure and the software Artemia 1.0®(Marnix Van Domme). In order to integrate the results of survival and growth, the criterion "total length" was introduced, *i.e.* total millimeters of *Artemia* per Falcon tube or mm/FT = numbers of survivors × mean individual length.

RPS value was determined in *Artemia* fed with various yeast strains accoprding to the following equation:

RPS (%) = (Percentage of surviving challenged *Artemia*)/(Percentage of surviving unchallenged *Artemia*) ×100

In experiment 2 and 3, the survival percentage in each treatment was determined daily. For this purpose, the number of live *Artemia* was registered before feeding (or adding any bacteria) by exposing each transparent Falcon tube to an incandescent light without opening the tube to preserve the axenity.

Values of larval survival (percentage) were arcsin transformed, while values of individual length (IL) and total biomass production (TBP) were logarithmic or square root transformed to satisfy normal distribution and homoscedasticity requirements. Differences in survival, RPS, IL and TBP of *Artemia* cultured in different conditions were investigated with analysis of variances (ANOVA) and Tukey's multiple comparison range. In all statistical analyses (SPSS 11.5 for Windows) the null hypothesis was rejected at the 0.05 level of probability.

3. Results

3.1. Artemia performance fed treated and untreated live yeast cells

Artemia nauplii were fed with 8 strains of baker's yeast (*Saccharomyces cerevisiae*) under gnotobiotic conditions. In all cases equal amounts of yeast cells were offered (Table 4.2).

The results presented in Table 4.3 (results obtained in experiment 1) show that independently of the chemical treatment, the yeast genetic background has a big influence on *Artemia* performance. Compared with WT yeast, total biomass production (TBP) of nauplii was significantly improved when isogenic yeast mutant strains were used as feed, due to both significant higher survival and/or individual length (IL) (Table 4.3). Among them, the mnn9 yeast strain supported the best nauplii performance.

Table 4.3 Experiment 1- average survival (%), individual length (IL) (mm) and total biomass production (TBP) (mm per Falcon tube-FT) of *Artemia* nauplii fed live yeast cells (harvested in the stationary growth phase and treated or untreated with 2ME) after 5 days: effect of yeast genetic background and chemical treatment on *Artemia* performance. Means were put together with the standard deviation (mean±S.D.). Each experiment was repeated twice A and B. Each feed was tested in four replicates. TN means treatment number. Values in the same column showing the same superscript letters are not significantly different (p_{Tukey}>0.05).

	Exp1	A			B		
TN		Survival (%)	IL (mm)	TBP (mm/FT)	Survival (%)	IL (mm)	TBP (mm/FT)
1	WT	43±7^{f}	1.4±0.1^{ef}	12.3±1.9^{i}	45±7^{e}	1.7±0.1^{ef}	12.3±1.9^{j}
2	WT-T	69±8de	1.5±0.1^{ef}	20.7±2.3^{g}	61±5^{d}	1.6±0.1^{ef}	20.7±2.3^{ghi}
3	mnn9	89±5^{ab}	3.6±0.1^{a}	64.0±3.5^{a}	83±3^{a}	3.5±0.1^{a}	64.0±3.5^{a}
4	mnn9-T	88±3^{ab}	3.6±0.4^{a}	63.0±2.1^{a}	84±8^{a}	3.6±0.1^{a}	63.0±2.1^{a}
5	mnn6	59±5^{ef}	1.3±0.1^{f}	14.8±1.2^{hi}	65±4^{cd}	1.4±0.1^{g}	14.8±1.2^{ij}
6	mnn6-T	88±3^{ab}	1.6±0.1^{de}	28.2±0.9^{def}	83±3^{a}	1.5±0.2^{efg}	28.2±0.9^{ef}
7	fks1	74±6^{cde}	1.6±0.1^{de}	23.3±2.0^{fg}	69±5^{bcd}	1.7±0.1^{de}	23.3±2.0^{fgh}
8	fks1-T	79±8^{bcd}	2.3±0.1^{c}	36.4±3.5^{c}	81±5^{a}	2.2±0.1^{c}	36.4±3.5^{d}
9	knr4	71±6de	1.4±0.1^{ef}	20.4±1.8^{g}	69±5^{bcd}	1.5±0.1^{fg}	20.4±1.8^{hi}
10	knr4-T	83±5^{abcd}	1.6±0.2^{de}	26.7±1.6^{ef}	78±3^{abc}	1.7±0.1^{de}	26.7±1.6^{efg}
11	kre6	85±4^{abc}	1.8±0.1^{d}	30.0±1.4^{de}	79±3^{ab}	1.7±0.1^{ef}	30.0±1.4^{e}
12	kre6-T	85±4^{abc}	2.6±0.1^{b}	44.9±2.2^{b}	88±7^{a}	2.2±0.1^{c}	39.3±2.9^{cd}
13	gas1	88±3^{ab}	2.6±0.1^{b}	46.0±1.5^{b}	84±3^{a}	2.8±0.1^{b}	46.0±1.5^{b}
14	gas1-T	90±4^{ab}	2.5±0.1^{bc}	44.5±2.0^{b}	81±5^{a}	2.8±0.1^{b}	44.5±2.0^{bc}
15	chs3	64±6^{e}	1.5±0.1^{de}	19.6±1.9^{gh}	66±8^{bcd}	1.6±0.1^{efg}	19.6±1.9^{hi}
16	chs3-T	91±3^{a}	1.6±0.1^{de}	32.7±0.9^{cd}	83±3^{a}	1.9±0.1^{d}	27.2±5.0^{ef}

Using 2ME-treated yeast cells as feed for *Artemia*, significantly improved *Artemia* biomass production either due to a considerable increase in survival (*e.g.* with WT, knr4 and chs3) or because of an improved IL (*e.g.* with fks1 and kre6) in respect to untreated yeast cells. Only by feeding treated mnn6 cells, improved TBP was due to both significant higher survival and higher individual length (IL) of *Artemia*. However when 2ME-treated mnn9 and gas1 cells were used to feed *Artemia,* no significant improvement in *Artemia* performance was observed in respect to untreated cells (see Table 4.3).

3.2. Artemia performance fed treated and untreated yeast cells and challenged

The results of the challenge tests, when supplying yeast cells and dead LVS3 (respectively 10 and 90% of the AFDW offered) as feed to the nauplii, are presented in Table 4.4 and 4.5. No significant difference was observed in *Artemia* survival until day 3 (before challenge test) (data not shown). Most of the unchallenged nauplii fed solely with dead LVS3 or both dead LVS3 and yeast cells survived until day 6 (68 % or higher). In most treatments, some challenged nauplii fed only with dead LVS3 or both dead LVS3 and untreated yeast cells (WT, mnn6, fks1, knr4, kre6) could survive until day 6 although with poor survival (18% or lower). Yet, exceptions occurred when the nauplii were fed both dead LVS3 and 2ME-treated mnn9, gas1 and chs3 yeast strains. Only mmn9 yeast cells (either treated or not) could provide high survival against the live pathogen until day 6 (Table 4.4 and 4.5, treatment 6 and 8). When 2ME-treated yeast cells were supplied to challenged nauplii, significantly improved *Artemia* survival, the RPS and the total biomass production values (in respect to untreated yeast cells) (Table 4.4 and 4.5 treatments: 22 vs 24, 26 vs 28 vs 30 vs 32) were noticed with some strains, namely kre6, gas1 and chs3. For all other cases 2ME-treatment did not enhance the survival, the RPS or the total biomass production of challenged *Artemia* (Table 4.4 and 4.5, treatments: 2 vs 4, 6 vs 8, 10 vs 12, 14 vs 16, 18 vs 20).

Table 4.4 Experiment 2: mean daily survival (%) of *Artemia* fed daily with dead LVS3 and yeast cell strains harvested in the stationary growth phase (treated or utreated with 2ME). The yeast cells constituted 10% of the total AFDW supplied (287 µg/FT). The challeng test was performed with *Vibrio campbellii* (VC) added on day 3. Each feed was tested in four replicates. TN means treatment number. RPS means relative percentage survival obtained in each treatment group (comparing the survival in the challenged treatment with the respective unchallenged (control)).The total amount of feed offered is equal to 2870µg AFDW/tube. Means were put together with the standard deviation (mean±SD.). Survival in the challenge test was compared directly to the survival of unchallenged *Artemia*. The effect of yeast chemical treatment on *Artemia* performance was demonstrated by comparing the effect of treated yeast cells in respect to untreated yeast cells on *Artemia* survival and growth. Values showing the same superscript letter are not significantly different ($p>0.05$).

TN	Exp 2	Survival (%)				IL (mm)	TBP (mm/FT)
		Day 4	Day 5	Day6	RPS (%)		
1	dead LVS3+WT10%	81 ± 5^{bcd}	79 ± 5^{abcd}	75 ± 4^{abc}	15^{g}	1.4 ± 0.1^{ghijk}	20.3 ± 1.1^{fghi}
2	dead LVS3+WT10%+VC	70 ± 4^{fgh}	20 ± 4^{j}	11 ± 3^{i}		1.4 ± 0.1^{fghij}	3.0 ± 0.7^{n}
3	dead LVS3+WT-T10%	84 ± 5^{abc}	81 ± 3^{abc}	79 ± 3^{abc}	20^{fg}	1.5 ± 0.1^{defghi}	23.7 ± 0.8^{ef}
4	dead LVS3+WT-T10%+VC	68 ± 6^{gh}	24 ± 6^{j}	16 ± 5^{ghi}		1.4 ± 0.1^{fghij}	4.6 ± 1.4^{lmn}
5	dead LVS3+mnn9 10%	91 ± 3^{a}	86 ± 3^{a}	80 ± 3^{a}	92^{a}	3.1 ± 0.3^{a}	49.9 ± 1.8^{a}
6	dead LVS3+mnn9 10%+VC	86 ± 3^{ab}	78 ± 3^{abcd}	74 ± 3^{abc}		2.8 ± 0.3^{a}	41.3 ± 1.4^{c}
7	dead LVS3+mnn9-T 10%	84 ± 3^{bc}	80 ± 4^{abc}	78 ± 3^{abc}	91^{a}	3.0 ± 0.2^{a}	47.1 ± 1.7^{ab}
8	dead LVS3+mnn9-T 10%+VC	83 ± 3^{bcd}	73 ± 3^{cde}	71 ± 3^{abcd}		2.7 ± 0.2^{a}	38.8 ± 1.4^{c}
9	dead LVS3+mnn6 10%	84 ± 3^{bc}	78 ± 3^{abcd}	70 ± 4^{bcd}	20^{fg}	1.4 ± 0.1^{ghijk}	19.7 ± 1.2^{ghi}
10	dead LVS3+mnn6 10%+VC	68 ± 5^{gh}	26 ± 3^{ij}	14 ± 3^{hi}		1.2 ± 0.1^{k}	3.4 ± 0.6^{n}
11	dead LVS3+mnn6-T 10%	83 ± 3^{bcd}	78 ± 3^{abcd}	73 ± 3^{abc}	25^{f}	1.5 ± 0.1^{defgh}	22.2 ± 0.8^{efg}
12	dead LVS3+mnn6-T 10%+VC	71 ± 3^{efgh}	30 ± 4^{ij}	18 ± 6^{ghi}		1.3 ± 0.1^{ijk}	4.6 ± 1.7^{lmn}
13	dead LVS3+fks1 10%	81 ± 3^{bcd}	76 ± 3^{abcd}	68 ± 3^{bcd}	26^{fg}	1.5 ± 0.1^{cdefgh}	20.6 ± 0.9^{fghi}
14	dead LVS3+fks1 10%+VC	70 ± 4^{fgh}	29 ± 5^{ij}	18 ± 3^{ghi}		1.3 ± 0.1^{hijk}	4.6 ± 0.8^{lmn}
15	dead LVS3+fks1-T 10%	84 ± 3^{bc}	79 ± 5^{abcd}	75 ± 4^{abc}	31^{ef}	1.5 ± 0.1^{defghi}	22.4 ± 1.2^{efg}
16	dead LVS3+fks1-T 10%+VC	71 ± 3^{efgh}	36 ± 6^{hi}	23 ± 3^{fgh}		1.4 ± 0.1^{ghijk}	6.3 ± 1.8^{klm}
17	dead LVS3+knr4 10%	85 ± 4^{ab}	78 ± 3^{abcd}	74 ± 5^{abcd}	13^{g}	1.3 ± 0.1^{jk}	19.54 ± 1.2^{ghi}
18	dead LVS3+knr4 10%+VC	68 ± 3^{gh}	24 ± 5^{j}	10 ± 4^{i}		1.3 ± 0.1^{hijk}	2.6 ± 1.1^{n}
19	dead LVS3+knr4-T 10%	86 ± 3^{ab}	81 ± 3^{abc}	75 ± 4^{abc}	24^{fg}	1.5 ± 0.1^{defghi}	22.5 ± 1.2^{efg}
20	dead LVS3+knr4-T 10%+VC	68 ± 3^{gh}	31 ± 6^{ij}	18 ± 6^{ghi}		1.4 ± 0.1^{ghijk}	4.8 ± 1.8^{lmn}
21	dead LVS3+kre6 10%	79 ± 3^{bcdef}	78 ± 3^{abcd}	74 ± 5^{abcd}	22^{fg}	1.5 ± 0.1^{efghij}	22.2 ± 1.4^{efg}
22	dead LVS3+kre6 10%+VC	65 ± 4^{h}	29 ± 5^{ij}	16 ± 3^{ghi}		1.4 ± 0.1^{fghijk}	4.6 ± 0.7^{lmn}
23	dead LVS3+kre6-T 10%	84 ± 3^{bc}	79 ± 5^{abcd}	75 ± 4^{abc}	51^{de}	1.7 ± 0.1^{bcd}	25.5 ± 1.4^{de}
24	dead LVS3+kre6-T 10%+VC	75 ± 4^{cdefgh}	51 ± 5^{fg}	38 ± 5^{f}		1.6 ± 0.1^{bcdef}	12.4 ± 1.6^{j}
25	dead LVS3+gas1 10%	84 ± 3^{bc}	73 ± 3^{cde}	69 ± 3^{bcd}	36^{ef}	1.8 ± 0.1^{b}	25.1 ± 0.9^{de}
26	dead LVS3+gas1 10%+VC	74 ± 5^{defgh}	45 ± 6^{gh}	25 ± 4^{fgh}		1.6 ± 0.1^{bcdefg}	7.9 ± 1.3^{kl}
27	dead LVS3+gas1-T 10%	86 ± 3^{ab}	84 ± 3^{ab}	79 ± 3^{abc}	67^{cd}	1.8 ± 0.1^{b}	28 ± 0.9^{d}
28	dead LVS3+gas1-T 10%+VC	78 ± 3^{bcdefg}	64 ± 5^{ef}	53 ± 5^{e}		1.6 ± 0.1^{bcdef}	17.3 ± 1.6^{i}
29	dead LVS3+chs3 10%	83 ± 3^{bcd}	75 ± 4^{bcde}	78 ± 3^{abc}	43^{de}	1.6 ± 0.1^{bcdef}	25.2 ± 1.4^{de}
30	dead LVS3+chs3 10%+VC	74 ± 5^{defgh}	49 ± 5^{gh}	34 ± 9^{f}		1.4 ± 0.1^{efghi}	9.7 ± 2.5^{jk}
31	dead LVS3+chs3-T 10%	86 ± 3^{ab}	84 ± 3^{ab}	81 ± 5^{ab}	74^{bc}	1.8 ± 0.1^{bc}	28.4 ± 1.7^{d}
32	dead LVS3+chs3-T 10%+VC	80 ± 4^{bcde}	68 ± 3^{de}	60 ± 6^{de}		1.6 ± 0.1^{bcdef}	19.7 ± 1.9^{ghi}
33	dead LVS3	83 ± 3^{bcd}	78 ± 3^{abcd}	70 ± 6^{bcd}	2^{fg}	1.4 ± 0.1^{efghi}	19.4 ± 1.6^{ghi}
34	dead LVS3+VC	68 ± 3^{gh}	28 ± 6^{ij}	15 ± 4^{ghi}		1.4 ± 0.1^{efghi}	4.1 ± 1.1^{mn}

Table 4.5 Experiment 3: mean daily survival (%) of *Artemia* fed daily with dead LVS3 and yeast cell strains harvested in the stationary growth phase (treated or untreated with 2ME). The yeast cells constituted 10% of the total AFDW supplied (287 µg/FT). The challeng test was performed with *Vibrio campbellii* (VC) added at day 3. Each feed was tested in four replicates. TN means treatment number. RPS means relative percentage survival obtained in each treatment group (comparing the survival in the challenged treatment with the respective unchallenged (control)). The total amount of feed offered is equal to 2870µg AFDW/tube. Means were put together with the standard deviation (mean±SD.). Survival in the challenge test was compared directly to the survival of unchallenged *Artemia*. The effect of yeast chemical treatment on *Artemia* performance was demonstrated by comparing the effect of treated yeast cells in respect to untreated yeast cells on *Artemia* survival and growth. Values showing the same superscript letter are not significantly different (p>*0.05)*.

TN	Exp. 3	Survival (%)				IL (mm)	TBP (mm/FT)
		Day4	Day 5	Day6	RPS (%)		
1	dead LVS3+WT10%	83±3^{abcde}	79±3^{ab}	74±3^{ab}	13^{f}	1.5±0.1^{fgh}	20.3±1.1^{ij}
2	dead LVS3+WT10%+VC	75±4^{defg}	18±6^{g}	10±6^{g}		1.4±0.1^{gh}	2.8±1.6^{m}
3	dead LVS3+WT-T10%	86±3^{ab}	83±3^{a}	76±3^{ab}	26^{ef}	1.6±0.1^{efgh}	23.8±0.8^{fghi}
4	dead LVS3+WT-T10%+VC	73±6^{fgh}	25±8^{fg}	20±8^{efg}		1.5±0.1^{efgh}	6.0±2.4^{m}
5	dead LVS3+mnn9 10%	88±3^{a}	83±3^{a}	81±3^{a}	92^{a}	3.0±0.2^{a}	48.0±1.5^{a}
6	dead LVS3+mnn9 10%+VC	89±3^{a}	75±4^{ab}	74±3^{ab}		2.6±0.2^{b}	38.8±1.3^{c}
7	dead LVS3+mnn9-T 10%	86±3^{ab}	81±8^{a}	79±5^{a}	89^{ab}	2.8±0.1^{ab}	43.8±2.7^{ab}
8	dead LVS3+mnn9-T 10%+VC	84±3^{abcd}	74±3^{ab}	70±4^{abc}		2.5±0.1^{b}	35.5±2.1^{c}
9	dead LVS3+mnn6 10%	83±3^{abcde}	78±6^{ab}	71±5^{ab}	21^{ef}	1.5±0.1^{efgh}	21.3±1.4^{hij}
10	dead LVS3+mnn6 10%+VC	75±4^{defg}	26±5^{fg}	15±4^{fg}		1.4±0.1^{gh}	4.2±1.1^{m}
11	dead LVS3+mnn6-T 10%	86±3^{ab}	80±4^{a}	78±3^{ab}	18^{f}	1.5±0.1^{efgh}	24.9±0.8^{fgh}
12	dead LVS3+mnn6-T 10%+VC	75±4^{defg}	23±5^{fg}	14±3^{fg}		1.4±0.2^{fgh}	4.0±0.7^{m}
13	dead LVS3+fks1 10%	79±3^{bcdefg}	74±5^{ab}	70±4^{abc}	20^{f}	1.6±0.1^{efgh}	20.7±1.2^{hij}
14	dead LVS3+fks1 10%+VC	75±4^{defg}	28±9^{fg}	14±6^{fg}		1.5±0.1^{gh}	3.9±1.8^{m}
15	dead LVS3+fks1-T 10%	84±3^{abcd}	80±4^{a}	76±3^{ab}	26^{ef}	1.6±0.1^{efg}	24.2±0.8^{fghi}
16	dead LVS3+fks1-T 10%+VC	78±3^{bcdefg}	28±5^{fg}	20±4^{efg}		1.5±0.1^{efgh}	6.0±1.2^{m}
17	dead LVS3+knr4 10%	84±3^{abcd}	76±3^{ab}	74±3^{ab}	20^{f}	1.5±0.1^{efgh}	22.4±0.8^{ghi}
18	dead LVS3+knr4 10%+VC	70±4^{gh}	28±5^{fg}	15±4^{fg}		1.4±0.1^{gh}	4.2±1.1^{m}
19	dead LVS3+knr4-T 10%	85±4^{abc}	78V3^{ab}	75±4^{ab}	15^{f}	1.5±0.1^{efgh}	22.8±1.2^{fghij}
20	dead LVS3+knr4-T 10%+VC	74±3^{efg}	23±5^{fg}	11±3^{fg}		1.4±0.1^{fgh}	3.2±0.7^{m}
21	dead LVS3+kre6 10%	84±3^{abcd}	80±4^{a}	74±3^{ab}	28^{ef}	1.7±0.1^{defg}	24.4±0.7^{fghi}
22	dead LVS3+kre6 10%+VC	74±5^{efg}	33±5^{ef}	21±8^{ef}		1.5±0.1^{efgh}	6.4±2.3^{lm}
23	dead LVS3+kre6-T 10%	88±3^{a}	80±7^{a}	74±6^{ab}	54^{cd}	1.8±0.1^{cde}	26.1±2.2^{efg}
24	dead LVS3+kre6-T 10%+VC	81±3^{abcdef}	54±4^{cd}	40±4^{d}		1.6±0.1^{defg}	13.1±1.3^{k}
25	dead LVS3+gas1 10%	89±3^{a}	81±5^{a}	76±5^{ab}	41de	1.9±0.1^{cd}	29.2±1.8^{de}
26	dead LVS3+gas1 10%+VC	76±5^{cdefg}	48±3de	31±5de		1.7±0.1^{defg}	10.3±1.6^{kl}
27	dead LVS3+gas1-T 10%	89±3^{a}	83±3^{a}	76±3^{ab}	74^{bc}	2.0±0.2^{c}	31.1±1.0^{d}
28	dead LVS3+gas1-T 10%+VC	79±3^{bcdefg}	65±9^{bc}	56±8^{c}		1.7±0.1^{defg}	18.6±2.5^{j}
29	dead LVS3+chs3 10%	85±4^{abc}	78±3^{ab}	71±3^{ab}	55^{cd}	1.7±0.1^{defg}	23.8±0.8^{fghi}
30	dead LVS3+chs3 10%+VC	79±3^{bcdefg}	45±7de	39±5^{d}		1.5±0.1^{efgh}	11.8±1.5^{k}
31	dead LVS3+chs3-T 10%	83±3^{abcde}	81±3^{a}	79±3^{a}	81^{ab}	1.7±0.1^{def}	27.0±0.9^{def}
32	dead LVS3+chs3-T 10%+VC	78±5^{bcdefg}	69±5^{abc}	64±9^{bc}		1.5±0.1^{fgh}	18.6±2.8^{j}
33	dead LVS3	86±3^{ab}	79±3^{ab}	75±4^{ab}	24^{ef}	1.4±0.1^{gh}	20.9±1.1^{hij}
34	dead LVS3+VC	63±3^{h}	28±3^{fg}	18±3^{fg}		1.4±0.1^{gh}	4.4±0.8^{m}

4. Discussion

In a model suggested by Lampen (1968), in yeast cell wall, mannoprotein molecules are mutually linked by phosphodiester bridges between their polysaccharide moieties. The phosphodiester cross-linking was supposed to form a physical barrier that retains the extracellular mannoprotein enzymes invertase, acid phosphatase, and others within the wall structure. Later studies revealed that, not only phosphodiester bridges but also disulfide linkages connect mannoprotein molecules through their protein moieties (Kidby and Davis, 1970). Farkas (1985) hypothesized that this cross-linking forms a barrier to penetration of extracellular glucanase into the internal glucan layer, which is the main structural constituent of the cell wall. As β-glucanase activity is detected in the digestive tract of *Artemia* (Coutteau *et al.,* 1990), a simple chemical treatment with 2ME, breaking disulfide linkages between mannoproteins molecules, giving rise to a more open structure in the cell wall (Zlotnik *et al.,* 1984), possibly facilitates the action of this enzyme on the yeast cell wall. Coutteau *et al.* (1990) reported that chemical treatment with 2ME on xenic baker's yeast significantly improved *Artemia* growth (as measured by individual length) (but not survival) when applied to exponentially-grown wild type yeast (average of 5.00 mm growth after 8 days) in comparison to untreated yeast cells (average of 2.93 mm growth after 8 days), but was ineffective in promoting digestion of the stationary-phase grown yeast cells (average of 2.63 mm growth after 8 days) in comparison to untreated yeast cells (average of 2.11 mm after 8 days). In the present study, similar growth was observed when *Artemia* nauplii were fed with stationary-grown and 2ME-treated WT cells. However, in contrary to previous results, *Artemia* survival was significantly improved when fed with 2ME-treated WT cells in comparison to untreated yeast cells (Table 4.3, treatment 1 vs 2).

In the current study, 2ME-yeast cells performed better as feed for *Artemia* in comparison to untreated yeast cells, when considering the total biomass production (TBP). This was due to an improved survival, mostly in combination with an improved individual growth (the latter with some exceptions, such as WT, knr4 and chs3 strains). However, feeding *Artemia* with treated mnn9 and gas1 strains did not improve the survival or total biomass production of *Artemia* in comparison to the respective

untreated yeast cells (Table 4.3, treatments: 3 vs 4 and 13 vs 14), probably because *Artemia* performance was already high.

When a limited amount of yeast cells were fed (only constituting 10% of the AFDW offered), there was no or very little improvement in survival and individual growth of *Artemia* in between 2ME-treated or untreated yeast cells. Also, at the survival level there was no difference between strains either treated or not. When compared with the individual growth of the WT (treated or not) only mnn9-fed *Artemia* performed considerably better.

Under such a feeding regime the effect of a *Vibrio campbellii* challenge was investigated. Only with three strains (kre6, gas1 and chs3) 2ME-treatment significantly enhanced survival. Hence, with these strains the enhanced resistance against VC was not concomitant with a better individual growth performance suggesting non-interference of general nutritional effects on the outcome of the challenge.

There is little background information available to explain this phenomenon, but apparently the type of gene mutations in these strains (kre6, gas1 and chs3) make changes in the yeast cell wall (*i.e.* changes in cell wall scaffolding or composition such as cell wall proteins probably influencing the density of disulfide bridges) enabling a positive action of 2ME treatment. This might increase cell wall β-glucans (considered to be an immunostimulant) availability to *Artemia*. Furthermore, it might be possible that the quality of β-glucans (*e.g.* molecular weight, three dimensional structure, type and frequency of branches and the ratio of β-1,3-glucans to β-1,6-glucans) is different between various yeast mutants used in the present study, which could interfere in glucan functions (Lipke and Ovalle, 1998).

It has been reported by De Nobel *et al.* (1994) that the yeast cell-wall mannoproteins can be divided into three groups according to the linkages that bind them to the structure of the cell wall: (i) non-covalently bond, (ii) covalently bond to the structural glucan, and (iii) disulfide bond to other proteins that are themselves covalently bond to the structural glucan of the cell wall. The amount of these three

groups of mannoproteins might be different in the various yeast strains. Because chemical treatment with 2ME presumably only affects the disulfide bonds, its effect must be dependent on the density of disulfide-bridges in cell wall-bound proteins present in mannoproteins, which in turn is dependent on the amount of mannoproteins. Unfortunately, there are no data available on the effect of the studied gene deletions on the density of mannoproteins and their linkage to each other and to glucans. A microarray study on altered gene expression in the fks1, gas1, knr4 and mnn9 deletion mutants, revealed that among the 300 genes verified, several genes coding for putative cell wall proteins were either strongly (*e.g.* CWP1 and CRH1) or weakly up or down regulated (Lagorce *et al.,* 2003). However, no strong correlation was found between the altered expression of those genes and the 2ME effect obtained in the VC challenge.

In conclusion, no negative effect of yeast treatment on *Artemia* performance was observed in this study. It has been shown that some yeast cell wall mutants (especially chs3 and to a minor extent kre6 and gas1) are predisposed for a beneficial effect of a 2ME treatment with respect to their potential to protect *Artemia* in a *Vibrio campbellii* challenge. However, the mechanism involved remains to be unravelled.

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CHAPTER V

The protective effect against *Vibrio campbellii* in *Artemia* nauplii by pure β-glucan and isogenic yeast cells differing in β-glucan and chitin content operated with a source-dependent time lag

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Abstract

In invertebrates the defence system to fight infectious diseases depends mainly on a non-specific or innate immune response, contrary to the vertebrate immune system. The use of natural immunostimulants that enhance the defence mechanism or the immune response of target organisms may be an excellent preventive tool against pathogens. Several strains of baker's yeast *Saccharomyces cerevisiae* have been found to be good immune enhancers. Previously, it was shown that small quantities of the mnn9 yeast cells and/or glucan particles could protect *Artemia* nauplii against the pathogenic bacterium *Vibrio campbellii* in the gnotobiotic *Artemia* challenge test. Apparently, the higher amount and/or availability of β-glucans and/or chitin present in mnn9 yeast strain might play an essential role in such protection. The present study reveals that these compounds could only provide protection against the pathogen when they were supplied to *Artemia* well in advance to the challenge (8 to 48h depending the source). Also the putative immunostimulant did not have a curative action. Moreover, short-time exposure of *Artemia* to mnn9 strain (priming) did not provide protection against the pathogen longer than 2 days. Hence, it is hypothesised that the mere stimulation of known biochemical pathways *e.g.* prophenoloxidase is not sufficient to explain the mechanisms involved in the observed immunostimulation obtained by β-glucans and/or mnn9 yeast in *Artemia* nauplii.

1. Introduction

Microbial diseases are a major threat to the sustainability of aquaculture, being responsible for massive mortalities occurring especially in the early life stages of aquatic organisms (Bachère,

2003). In these larval stages, most organisms, including vertebrates, cannot rely on an acquired immune system to combat disease, but have to rely on the innate immune system (Arala-Chaves and Sequeira, 2000; Kurtz and Franz, 2003; Little and Kraaijeveld, 2004). Infact, fish larvae and invertebrates are not equipped with cells that are analogous to antibody producing lymphocytes in vertebrates. According to Raa (2000), invertebrates are apparently entirely dependent on non-specific immune mechanisms to cope with infections, as they lack the specific immunological "memory" that is found in fish and warm-blooded animals. Yet, a recent study in the copepod *Macrocyclops albidus* showed that the defense system of this invertebrate species reacted more efficiently after a previous encounter with an antigenically similar parasite, implying that a specific memory may exist (Kurtz and Franz, 2003). Indeed, many studies have now explicitly shown that primary exposure (immunological priming) to pathogens or even micro-organisms cell wall components may be prophylactic, providing protection during secondary encounters (Moret and Siva-Jothy, 2003). Nowadays, the use of preventive and environment-friendly approaches such as probiotics, immunostimulatns, antibacterial peptides and

quorum sensing systems is becoming increasingly important in aquaculture (Bachère, 2003; Raa, 2000; Sakai, 1999; Verschuere *et al.,* 2000; Defoirdt *et al.,* 2005; Alabi *et al.,* 1999; Itami *et al.,* 1998). However, the application of such technologies must be based on thorough understanding of the mechanisms involved and the putative consequences. An essential part of that understanding can be provided by studies looking in detail at host-microbial interactions. A key experimental approach to study these interactions is to first define the functioning of the host in the absence of bacteria and then to evaluate the effect of adding a single or defined population of microbes, or certain compounds (*i.e.,* under axenic or gnotobiotic conditions) (Teunissen *et al.,* 1998; Anderson, 1992; Sung *et al.,* 1996). Marques *et al*. (2004a) have recently developed and validated the usefulness of an *Artemia* gnotobiotic test system allowing to study the effect of food composition as well as the host-microbial interaction on survival and growth of *Artemia* in the presence or absence of a pathogen.

The use of specific biological compounds (immunostimulants) that enhance immune responses of target organisms, rendering animals more resistant to diseases may be an excellent preventive tool against pathogens (Anderson, 1992).

Several immunostimulants have been used in vertebrate and invertebrate culture, to induce protection against a wide range of diseases: *i.e.* β-glucans (Sung *et al.,* 1996; Sritunyalucksana *et al.,* 1999; Burgents *et al.,* 2004; Misra *et al.,* 2004), chitin (Anderson and Siwicki, 1994; Song and Huang, 1999; Wang and Chen, 2005), mannoproteins (Tizard *et al.,* 1989), lipopolysaccharides (Takahashi *et al.,* 2000), peptidoglycans (Itami *et al.,* 1998; Boonyaratpalin *et al.,* 1995) and dead bacteria (Alabi *et al.,* 1999; Keith *et al.,* 1992; Vici *et al.,* 2000).

The present study investigates the use of baker's yeast and glucan particles in gnotobiotic *Artemia* to promote the immune system against the pathogenic bacteria *Vibrio campbelli*. For that purpose, commercially available particles of glucans obtained from *S. cerevisiae* and intact cells of two strains of live baker's yeast were offered as feed (under various feeding regimes) to gnotobiotic *Artemia* with the primary aim to study the dynamics of the induction of the protective response by these particles.

2. Material and methods

2.1. Axenic culture of yeast

Two different strains of baker's yeast (*Saccharomyces cerevisiae*) were used as feed for *Artemia*: the wild-type strain (WT) and its mnn9 isogenic mutant which has a null mutation resulting mainly in a lower concentration of protein-bound mannose in the outer layer of the cell wall (Marques *et al.,* 2004b). Both strains were provided by EUROSCARF (University of Frankfurt, Germany).

Yeasts cultures were performed according to procedures previously described by Marques *et al.* (2004b) using minimal Yeast Nitrogen Base medium (YNB) to culture mnn9 yeast (mnn9-YNB) and a complete Yeast Extract Peptone Dextrose medium (YEPD) to culture WT yeast (WT-YEPD). The WT strain was harvested by centrifugation (± 800 × g for 10min) in the stationary growth phase (after 3 days;"stat.yeast") while mnn9 yeast was harvested in the exponential growth phase (after 20

h; "exp.yeast"). This results typically in a cell-wall composition of 1.2% chitin, 47.3% mannose and 51.5 % glucans in the WT yeast, while the mnn9 yeast mutant contained 8.7% chitin, 16.3% mannose and 75.0 % glucans (Marques *et al.,* 2004a).

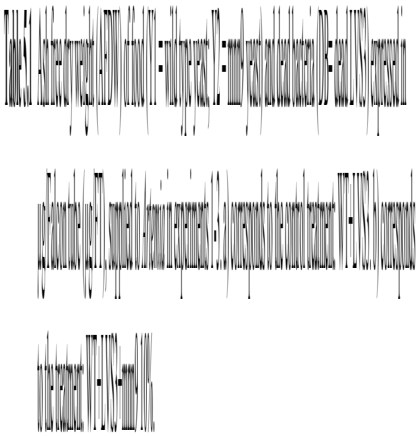
All handling was performed in a laminar flow hood and all necessary tools were previously autoclaved at 120 °C for 20 min to ensure sterility. Yeasts were resuspended in filtered and autoclaved seawater (FASW, 0.2 µm) and their densities were determined by measuring twice the cell concentration, using a Bürker haemocytometer. Suspensions were stored at 4 °C and provided to *Artemia* until the end of each experiment. The following two feeds were chosen because of the protection they provide against pathogenic bacteria: no protection (WT yeast) and protection (mnn9 yeast) (Marques *et al.,* 2005).

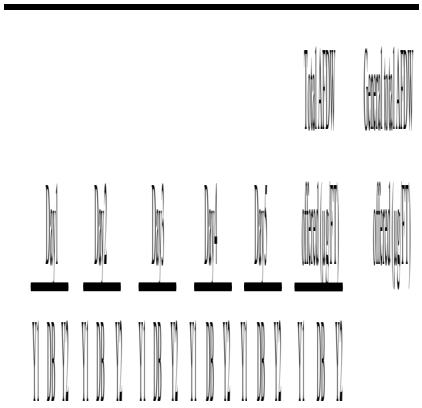
2.2. Bacterial strains and growth conditions

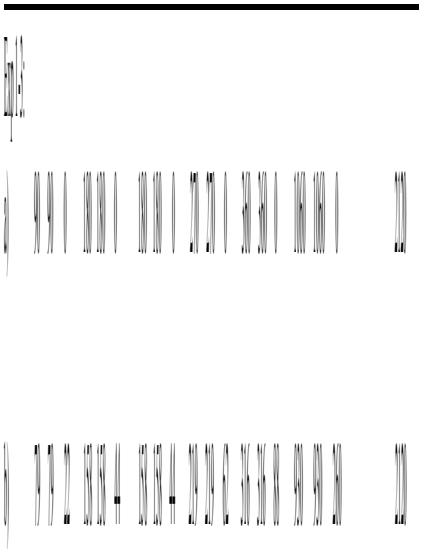
Two bacterial strains were used, *i.e. Aeromonas hydrophila* strain LVS3 (Marques *et al.,* 2005; Soto-Rodriguez *et al.,* 2003; Gomez-Gil *et al.,* 2004) for its positive effect on *Artemia* performance when fed at sub-optimal concentration and *Vibrio campbellii* strain LMG21363 (VC) for its pathogenic effect towards *Artemia* and shrimp (Soto-Rodriguez *et al.,* 2003; Gomez-Gil *et al.,* 2004; Coutteau *et al.,* 1990; Aguilar-Uscanga and François, 20003). The two bacterial strains were cultured and harvested according to procedures described by Marques *et al.* (2005). Bacteria were resuspended in FASW and their densities determined by spectrophotometry (OD_{550}), assuming that an optical density of 1.000 corresponds to 1.2×10^9 cells/ml, according to the McFarland standard (Biomerieux, Marcy l'Etoile, France). At day 3, challenge tests were performed with live VC, i.e the pathogen was added to each replicate at a density of 5×10^6 cells/ml. Dead LVS3 was provided to *Artemia* using aliquots of autoclaved (120°C for 20 min) and centrifuged-concentrated bacteria. After autoclaving, bacteria were plated to check if they were effectively killed by this method (Marques *et al.,* 2004a). Dead and live bacterial suspensions were stored at 4°C until the end of each experiment.

2.3. Artemia gnotobiotic culture

Experiments were performed with *Artemia franciscana* cysts, originating from Great Salt Lake, Utah- USA (EG ® type, INVE Aquaculture, Belgium). Bacteria-free cysts and nauplii were obtained using the procedures described by Sorgeloos *et al.* (1986) and Marques *et al.* (2004a). After hatching, 20 axenic nauplii (Instar II) were transferred by pipette to Falcon tubes containing 30 ml FASW as well as the amount of feed scheduled for day 1 (see further for feeding schedule). The daily feeding schedule was adopted from Coutteau *et al*. (1990) and Marques *et al.* (2004a) and was intended to provide *ad libitum* rations but avoiding excessive feeding in order not to affect the water quality in the test tubes. The total AFDW of yeast and bacteria added to *Artemia* in experiments 1, 2 and 3 is presented in Table 5.1. In experiment 4, nauplii were fed with WT and/ or mnn9 yeast cells (only for 8 h) with the amount of feed scheduled for day 1 (adopted from Coutteau *et al.* (1990) in combination with dead LVS3 at a density of 10^7 cells/ml/day. Falcon tubes were placed on a rotating rod (4 cycles per min), exposed to constant incandescent light (41μEm^{-2}) at 28°C. Tubes were transferred once per day to the laminar flow for feeding.







2.4. Method used to verify axenity

Axenity of feed, decapsulated cysts and *Artemia* cultures was checked at the end of each experiment using a combination of plating (MA) and live counting (using tetrazolium salt MTT staining) following the procedure described by Marques *et al.* (2004a). In challenge treatments, the axenity of *Artemia* culture was always checked before challenge using the same methods. Contaminated culture tubes were not considered for further analysis and the treatment was repeated.

2.5. Glucan particles

A suspension of glucan particles was prepared and offered to *Artemia* nauplii according to a procedure described by Marques *et al*. (2006). For that purpose, pure insoluble glucan particles obtained from the baker's yeast *Saccharomyces cerevisiae* (Sigma, 100 mg) were aseptically transferred to sterile 50 ml Falcon tubes and homogeneously suspended in FASW. Contaminations were checked by plating the suspension on Marine Agar (100 µl, $n = 2$). Absence of bacterial growth was monitored after incubating the plates for 5 days at 28 °C. No bacterial growth was detected on marine agar after the incubation period. An optical laser particle size analyser (Mastersizer MSX-17, Malvern Instruments Ltd., Malvern, Works, UK; resolution 0.05–900 µm) was used to determine the diameter of the glucan particles present in the suspension, using the software Malvern Mastersizer S version 2.19® (Malvern Instruments Ltd., Malvern, Works, UK). Only 12.11% of the particles had a diameter lower than 50 µm (which is considered to be the upper limit for possible uptake by *Artemia*). This amount of glucan was calculated based on the feeding regime for WT yeast as published by Marques *et al.* (2004a, 2005) taking into account that these WT yeast cells contain 51.5% glucan in their cell wall. In order to provide 130 µg/tube or 26 µg/tube/day of ingestible glucan it was necessary to provide ±1 mg/tube or ±200 µg/tube/day of glucan. The suspension of glucans was stored at 4 °C until the end of the experiment being provided daily in equal portions per day to *Artemia* (26 µg/tube/day).

2.6. Experimental design

The experimental design for the 4 culture tests is schematized in Fig. 5.1 and 5.2. In experiment 1, small amounts of glucan particles (±200 µg/tube/day) or mnn9 yeast cells (10% of the total amount of food (in AFDW), offered in equal parts per day, in order to avoid the possibility that those yeast cells could be used as major feed source by *Artemia*) were daily added as food to the *Artemia* using a combination of WT yeast and dead LVS 3 (WT + LVS3). At day 3, challenge tests were performed by adding VC to each replicate at a concentration of approximately 5×10^6 cells/ml.

Fig. 5.1 Experimental design of the experiments (Exp) 1 to 4 performed. Legend: a-m) corresponds to the treatments performed; F- feed provided (mixture of WT yeast and dead LVS3); G – glucan particles (added at different time intervals); P – pathogen (*Vibrio campbellii)*, added on day 3 in Exp 1-3 and 8 hours after start feeding in Exp 4); Y2 – mnn9 yeast cell (added at10% of AFDW at different periods in Exp 1-3); NF – means no feed added; DB – dead bacterium LVS3; Y1 – WT yeast (added only for 8 hours).

		Day 1 Start	Day 2	Day 3	Day 4	Day 5	Day 6 Harvest
Exp 1-3	a)	F →	F →	F →	F →	F →	
	b)	F →	F →	F+P →	F →	F →	
	c)	F+G →	F+G →	F+G →	F+G →	F+G →	
	d)	F+G →	F+G →	F+G+P →	F+G →	F+G →	
	e)	F →	F+Y2 →	F+Y2 →	F+Y2 →	F+Y2 →	
	f)	F →	F+Y2 →	F+Y2+P →	F+Y2 →	F+Y2 →	
Exp 4	g)	NF →	NF →	Harvest			
	h)	DB →	DB →	Harvest			
	i)	DB →	DB →	Harvest			
	j)	Y1 (0-8h)+DB+P(8h) →	DB →	Harvest			
	k)	Y1 (0-8h)+DB+P(8h) →	DB →	Harvest			
	l)	Y2 (0-8h)+DB+P(8h) →	DB →	Harvest			
	m)	Y2 (0-8h)+DB+P(8h) →	DB →	Harvest			

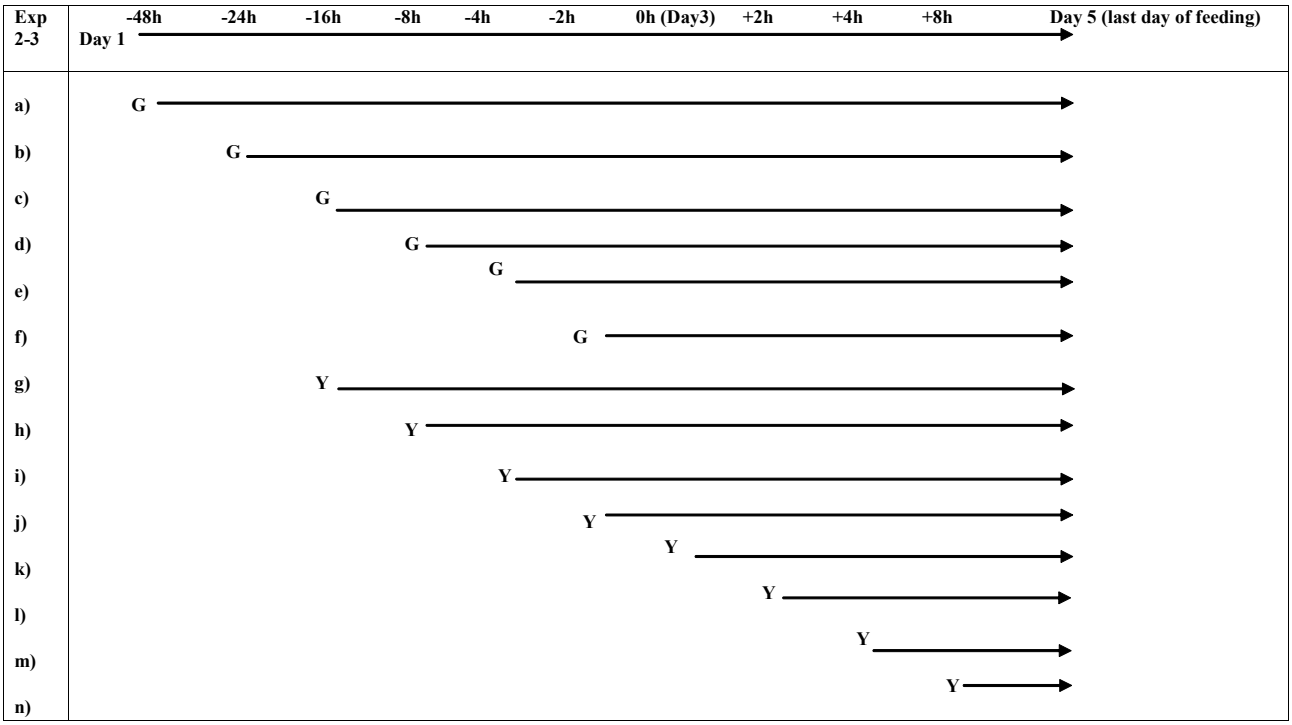


Fig 5.2 Feeding scheme of glucan particles (G) or mnn9 yeast cells (Y) applied in experiment 2 and 3. Glucan or mnn9 yeast (added at 10% of total AFDW) were offered to *Artemia* at various time intervals (since 48h before challenge (-48h) until 8h after challenge (+8h)). Nauplii were challenged with *Vibrio campbellii* on day 3. Feeding continued until end of experiment (day5).

In experiment 2 and 3, the same feeding regime was applied as in experiment 1, except for the daily addition of mnn9 yeast cells and/or glucan particles, which was started at different time points prior to the challenge (see Fig. 5.2). As a result, *Artemia* were daily fed with the major food source (WT yeast + dead LVS3) in combination with the mnn9 yeast cells and/or glucan particles added *e.g* from 48h before (-48h) until 8h after (+8h) the challenge test. In experiment 4, *Artemia* nauplii were fed with WT or mnn9 yeast cells (same feeding regime for day1 -adopted from Coutteau *et al.* (1990)- but only for 8h) (see Fig. 5.1). The nauplii were subsequently washed (axenically by serial dilution with FASW) to remove all remaining yeast cells and then fed during 2 days with dead LVS3 at a density of 10^7 cells/ml/day. At the same time the nauplli were challenged with VC at a density of 5×10^6 cells/ml.

2.7. Survival and growth of Artemia

Survival and growth of the *Artemia* nauplii were determined according to the procedures described by Marques *et al.* (2004a). For each treatment the survival percentage was determined daily *i.e.* the number of live *Artemia* was registered before feeding or adding any bacteria by exposing each transparent Falcon tube to an incandescent light without opening the tube as to maintain the gnotobiotic environment. At the end of each experiment (day 6 after hatching), live *Artemia* were fixed with lugol's solution to measure individual length (IL), using a dissecting microscope equipped with a drawing mirror, a digital plan measure and the software Artemia 1.0® (courtesy Marnix Van Damme). As a criterion that combines both the effects of survival and IL, the total biomass production (TBP) was determined according to the following equation:

TBP (millimeters per Falcon tube - mm/FT) = number of survivors × mean IL

2.8. Statistics

Values of larval survival (percentage) were arcsin transformed, while values of IL and TBP were logarithmic or square root transformed to satisfy normal distribution and homoscedasticity requirements. Differences in survival, IL and TBP of *Artemia* cultured in different conditions were investigated with analysis of variances (ANOVA) and Tukey's multiple comparison range. All statistical analyses were tested at the 0.05 level of probability, using the software SPSS 11.5 for Windows.

3. Results

In experiment 1, *Artemia* performance was compared to results previously obtained by Marques *et al.*(2006) in order to evaluate reproducibility. In this study similar results were obtained although the *Artemia* performance (mainly survival) in the challenge test was lower than expected (especially within glucan treated *Artemia*). Results presented in Table 5.2 indicate that unchallenged nauplii fed soley with a mixture of dead LVS3 and WT yeast survived until day 6 with low total biomass production (TBP). The addition of glucans to unchallenged *Artemia* fed with a combination of dead

LVS3 and WT did not improve *Artemia* performance. Yet, the addition of 10% of mmn9 to unchallenged and to nauplii fed with a mixture of dead LVS3 and WT yeast significantly improved TBP in comparison to nauplii fed only the combination of dead LVS3 and WT yeast, mainly as a result of improved growth (individual length). Nauplii challenged with VC and only fed with the mixture (Table 5.2, treatment 2) presented significantly lower TBP in comparison to unchallenged nauplii (Table 5.2, treatment 1) because of a significant drop in survival. Yet, significantly higher survival rates and TBP were obtained when glucan or 10% of mnn9 yeast were provided to challenged nauplii in comparison to challenged nauplii not supplied with theses products (Table 5.2 treatment 4 and 6 vs treatment 2).

Table 5.2 Experiment 1- Mean survival (%), individual length (IL) (mm) and total biomass production (mm per Falcon tube- FT) of *Artemia* fed various feed combinations on day6. Particles of glucan (G) and /or mnn9 yeast cells (constituted 10% of the total AFDW) were supplied daily together with a combination of WT yeast and dead LVS3 (WT+LVS3). The challenge test was performed with *Vibrio campbellii* (VC) added on day 3 (D3). The first column on the table refers to the type of the treatment (see Fig. 5.1 and 5.2). Values are presented with the respective standard deviation (mean±SD). Values in the same column showing the same superscript letter are not significantly different (p *Tukey*> 0.05). This experiment was repeated twice, A and B. TN corresponds to treatment number.

		A			B		
TN	Experiment 1	Survival (%)	IL (mm)	TBP (mm/FT)	Survival (%)	IL (mm)	TBP (mm/FT)
1	WT+ dead LVS3	72 ± 10^{bc}	1.86 ± 0.06^{b}	26.70 ± 2.44^{bc}	64 ± 8^{bc}	1.70 ± 0.02^{b}	21.76 ± 2.32^{b}
2	WT+ dead LVS3+VC D3	9 ± 3^{a}	1.49 ± 0.09^{cd}	2.73 ± 1.06^{a}	14 ± 4^{a}	1.50 ± 0.02^{bc}	4.20 ± 0.55^{a}
3	WT+ dead LVS3+ mnn9 10%	80 ± 6^{bd}	2.51 ± 0.46^{a}	40.22 ± 5.20^{d}	72 ± 8^{bd}	2.63 ± 0.08^{a}	37.87 ± 3.94^{c}
4	WT+ dead LVS3+ mnn9 10% +VC D3	68 ± 4^{bc}	2.44 ± 0.02^{ab}	33.16 ± 2.36^{cd}	64 ± 5^{bc}	2.56 ± 0.43^{a}	32.75 ± 3.20^{cd}
5	WT+ dead LVS3+ G	70 ± 8^{bd}	1.33 ± 0.06^{cd}	18.59 ± 2.08^{cd}	65 ± 4^{bc}	1.40 ± 0.08^{cd}	18.14 ± 2.35^{cd}
6	WT+ dead LVS3+ G +VC D3	60 ± 5^{bc}	1.39 ± 0.22^{cd}	16.64 ± 2.32^{bc}	57 ± 6^{bd}	1.28 ± 0.03^{cde}	14.56 ± 2.94^{d}

In experiment 2 and 3, the dynamics of induction of the protective effect of glucan and/or mnn9 yeast on *Artemia* in a challenge test was studied. The results presented in Table 5.3 show that in order to be able to induce optimum protection against the pathogen it was necessary to expose *Artemia* to glucan at least 48h prior to challenge (see also Fig. 5.3), while mnn9 yeast cells could provide optimum protection when included in the feed at least 8h before challenge (Table 5.4: treatment 5 and 6) (see also Fig. 5.4). The results indicate that any shorter exposure to glucan and/or mnn9 yeast resulted in significantly lower TBP of *Artemia* in the VC challenge test due to reduced survival and IL (*e.g.* in mnn9 treatments) or mainly due to reduced survival (*e.g.* in glucan treatments) in comparison to nauplii fed with these compounds for sufficiently long periods (treatment 3 vs treatment 4, 6, 8, 10, 12 and 14 in Table 5.3 and treatment 3 and 5 vs treatment 8, 10, 12, 14, 16 and 18 in Table 5.4).

Table 5.3 Experiment 2- Mean survival (%), individual length (mm) and total length (mm/ per Falcon tube- FT) of *Artemia* fed various feed combinations on day 6. Particles of glucan (G) were supplied daily at different time intervals together with a combination of WT yeast and dead LVS3 (WT+LVS3). The challenge test was performed with *Vibrio campbellii* (VC) added on day 3 (D3). The first column on the table refers to the type of the treatment (see Fig. 5.1 and 5.2). Values are presented with the respective standard deviation (mean±SD). Values in the same column showing the same superscript letter are not significantly different (p T*ukey*>0.05). This experiment was repeated twice, A and B. TN corresponds to treatment number.

		A			B		
TN	Experiment 2	Survival (%)	IL (mm)	TBP (mm/FT)	Survival (%)	IL (mm)	TBP (mm/FT)
1	WT+dead LVS3	66 ± 6^{bc}	1.80 ± 0.14^{a}	23.78 ± 2.44^{a}	68 ± 9^{a}	1.78 ± 0.08^{a}	23.96 ± 2.85^{a}
2	WT+dead LVS3+VC D3	10 ± 4^{f}	1.39 ± 0.09^{cd}	2.77 ± 1.18^{f}	11 ± 3^{c}	1.34 ± 0.27^{de}	2.99 ± 0.76^{de}
3	WT+dead LVS3+ G (-48h)	63 ± 10^{bc}	1.37 ± 0.06^{cd}	17.10 ± 2.15^{ab}	55 ± 7^{a}	1.31 ± 0.80^{de}	14.48 ± 2.67^{ab}
4	WT+dead LVS3+ G (-48h) +VC D3	56 ± 5^{c}	1.36 ± 0.03^{cd}	15.26 ± 1.47^{bc}	53 ± 6^{a}	1.22 ± 0.11^{e}	12.92 ± 2.51^{b}
5	WT+dead LVS3+ G (-24h)	70 ± 4^{ab}	1.58 ± 0.10^{abc}	22.12 ± 2.49^{ab}	60 ± 9^{a}	1.45 ± 0.04^{cde}	17.33 ± 2.28^{ab}
6	WT+dead LVS3+ G (-24h) +VC D3	40 ± 4^{d}	1.31 ± 0.10^{d}	10.50 ± 1.37^{cd}	28 ± 3^{b}	1.32 ± 0.06^{e}	7.28 ± 0.93^{c}
7	WT+dead LVS3+ G (-16h)	76 ± 3^{a}	1.77 ± 0.03^{a}	26.92 ± 1.06^{a}	55 ± 7^{a}	1.56 ± 0.04^{abc}	17.55 ± 2.26^{ab}
8	WT+dead LVS3+ G (-16h) +VC D3	34 ± 3^{de}	1.41 ± 0.19^{bcd}	9.61 ± 1.98^{de}	29 ± 6^{b}	1.26 ± 0.13^{de}	7.21 ± 1.69^{c}
9	WT+dead LVS3+ G (-8h)	74 ± 8^{ab}	1.78 ± 0.12^{a}	26.26 ± 3.97^{a}	59 ± 8^{a}	1.68 ± 0.06^{ab}	19.77 ± 2.85^{ab}
10	WT+dead LVS3+ G (-8h) +VC D3	24 ± 5^{e}	1.33 ± 0.14^{cd}	6.37 ± 1.94^{e}	24 ± 5^{b}	1.46 ± 0.17^{bcd}	7.06 ± 2.29^{c}
11	WT+dead LVS3+ G (-4h)	70 ± 3^{ab}	1.75 ± 0.04^{a}	24.47 ± 1.58^{a}	66 ± 5^{a}	1.74 ± 0.08^{a}	23.08 ± 1.64^{a}
12	WT+dead LVS3+ G (-4h) +VC D3	25 ± 4^{e}	1.40 ± 0.04^{bcd}	7.01 ± 1.36^{de}	19 ± 3^{bc}	1.40 ± 0.14^{cde}	5.20 ± 0.43^{c}
13	WT+dead LVS3+ G (-2h)	69 ± 3^{abc}	1.64 ± 0.01^{ab}	22.58 ± 0.78^{ab}	63 ± 10^{a}	1.82 ± 0.10^{a}	22.83 ± 4.61^{a}
14	WT+dead LVS3+ G (-2h) +VC D3	25 ± 7^{e}	1.36 ± 0.15^{cd}	6.90 ± 2.50^{e}	11 ± 5^{c}	1.44 ± 0.10^{bcde}	3.20 ± 1.32^{d}

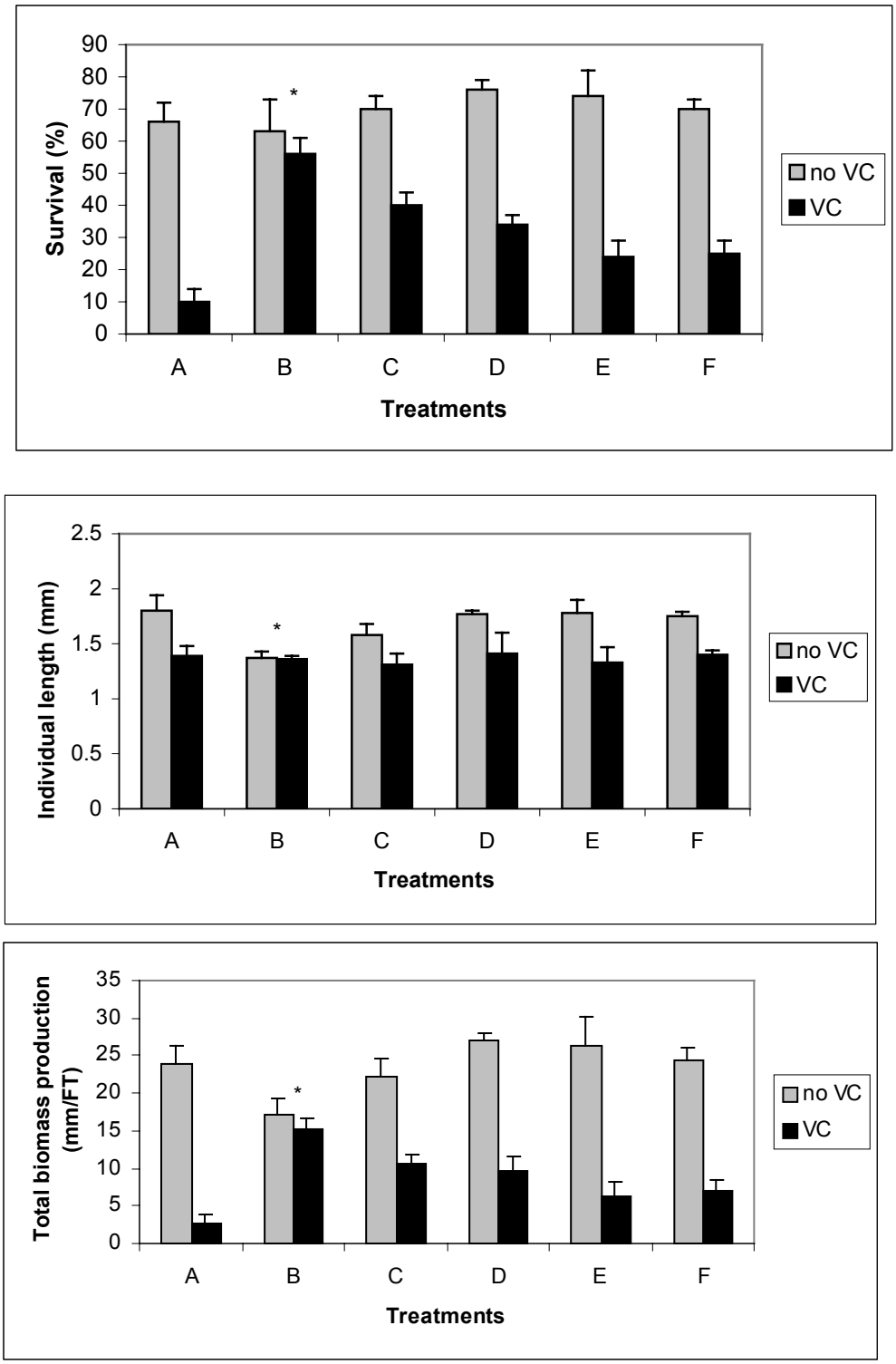


Fig. 5.3 Histogram of the average *Artemia* survival, individual length and total biomass production (mm/FT) when fed various feed combinations on day 6. Particles of glucan (G) were supplied daily at different time intervals (before challenge test ("-h") together with a combination of WT yeast and dead LVS3 (WT+LVS3). As a control, nauplii were not supplied with glucan (treatment A). A) corresponds to treatment: WT+dead LVS3. B) corresponds to treatment: WT+dead LVS3+G (-48h). C) corresponds to treatment: WT+dead LVS3+G (-24h). D) corresponds to treatment: WT+dead LVS3+G(-16h). E) corresponds to treatment: WT+dead LVS3+G(-8h). F) corresponds to WT+dead LVS3+G (-4h). Grey bars represent results of unchallenged nauplii while black bars reperesents nauplii challenged with *Vibrio campbellii* (VC) added on day 3 . Asterisks mean no significant differences between two bars (p T*ukey*>0.05).

Table 5.4 Experiment 3- Mean survival (%), individual length (IL) (mm) and total biomass production (mm per Falcon tube- FT) of *Artemia* fed various feed combinations on day 6. The mnn9 yeast cells (constituted 10% of the total AFDW) were supplied daily at different time intervals together with a combination of WT yeast and dead LVS3 (WT+LVS3). As a control, nauplii were not supplied with mnn9 yeast cells. The challenge test was performed with *Vibrio campbellii* (VC) added on day 3 (D3). The first column on the table refers to the type of the treatment (see Fig. 5.1 and 5.2). Values are presented with the respective standard deviation (mean±SD). Values in the same column showing the same superscript letter are not significantly different ($pTukey>0.05$). This experiment was repeated twice, A and B. TN corresponds to treatment number.

		A			B		
TN	Experiment 3	Survival (%)	IL (mm)	TB (mm FT)	Survival (%)	IL (mm)	TB (mm FT)
1	Yeast LVS3	66±6^{bc}	1.8±0.1^{c}	23.8±2.4^{bc}	68±9^{bc}	1.78±0.08^{cdef}	23.6±2.8^{bcd}
2	Yeast LVS3+VC3	10±4^{f}	1.3±0.0^{d}	2.7±1.8^{g}	11±5^{g}	1.34±0.2^{g}	2.9±0.6^{g}
3	Yeast LVS3+mnn9 (16)	75±5^{ab}	2.0±0.2^{a}	42.3±4.8^{a}	79±6^{ab}	2.3±0.2^{a}	44.6±3.2^{a}
4	Yeast LVS3+mnn9 (16)+VC3	66±6^{bc}	2.2±0.1^{c}	28.4±2.8^{ab}	62±4^{bc}	2.3±0.2^{ab}	26.6±2.8^{bcd}
5	Yeast LVS3+mnn9 (8)	79±5^{a}	2.7±0.3^{a}	43.8±6.6^{a}	75±4^{ab}	2.6±0.3^{a}	39.5±2.7^{ab}
6	Yeast LVS3+mnn9 (8)+VC3	61±5^{bc}	2.1±0.1^{bc}	25.9±2.1^{ab}	64±5^{bc}	2.3±0.1^{bcd}	25.9±3.7^{bcd}
7	Yeast LVS3+mnn9 (4)	76±5^{ab}	2.8±0.2^{a}	42.6±5.8^{a}	80±4^{a}	2.6±0.1^{a}	42.4±2.8^{a}
8	Yeast LVS3+mnn9 (4)+VC3	53±6^{cd}	2.1±0.1^{c}	22.6±4.1^{bc}	58±6^{c}	1.9±0.1^{bcde}	22.8±2.3^{cd}
9	Yeast LVS3+mnn9 (2)	78±6^{ab}	2.8±0.1^{a}	43.1±4.2^{a}	80±4^{a}	2.8±0.2^{a}	42.6±2.8^{a}
10	Yeast LVS3+mnn9 (2)+VC3	54±8^{c}	2.1±0.1^{c}	22.6±4.2^{bc}	55±4^{c}	2.1±0.2^{bc}	23.0±4.5^{cd}
11	Yeast LVS3+mnn9 (1)	80±7^{a}	2.5±0.1^{ab}	41.2±2.6^{a}	78±6^{ab}	2.1±0.1^{abc}	32.6±3.9^{abcd}
12	Yeast LVS3+mnn9 (1)+VC3	34±6de	1.9±0.1^{c}	12.9±1.6^{cd}	30±4^{d}	1.6±0.2^{cdef}	10.1±1.8^{e}
13	Yeast LVS3+mnn9 (2)	80±7^{a}	2.8±0.1^{ab}	41.2±3.8^{a}	79±5^{a}	2.2±0.1^{ab}	34.8±3.2^{abcd}
14	Yeast LVS3+mnn9 (2)+VC3	25±9^{e}	1.9±0.1^{c}	9.6±4.1^{d}	21±5^{d}	1.5±0.2^{ef}	6.7±1.9^{ef}
15	Yeast LVS3+mnn9 (4)	79±5^{a}	2.5±0.1^{ab}	39.8±1.4^{a}	80±7^{a}	2.2±0.1^{ab}	35.4±2.6^{abc}
16	Yeast LVS3+mnn9 (4)+VC3	24±9^{e}	1.8±0.1^{c}	8.8±3.8^{d}	21±6^{d}	1.5±0.1^{ef}	6.7±2.4^{ef}
17	Yeast LVS3+mnn9 (8)	76±6^{ab}	2.8±0.0^{a}	43.2±2.2^{a}	78±6^{ab}	2.1±0.1^{ab}	33.7±2.6^{abcd}
18	Yeast LVS3+mnn9 (8)+VC3	26±9^{e}	1.8±0.1^{c}	9.7±3.2^{d}	20±4^{d}	1.6±0.2^{def}	6.6±1.4^{ef}

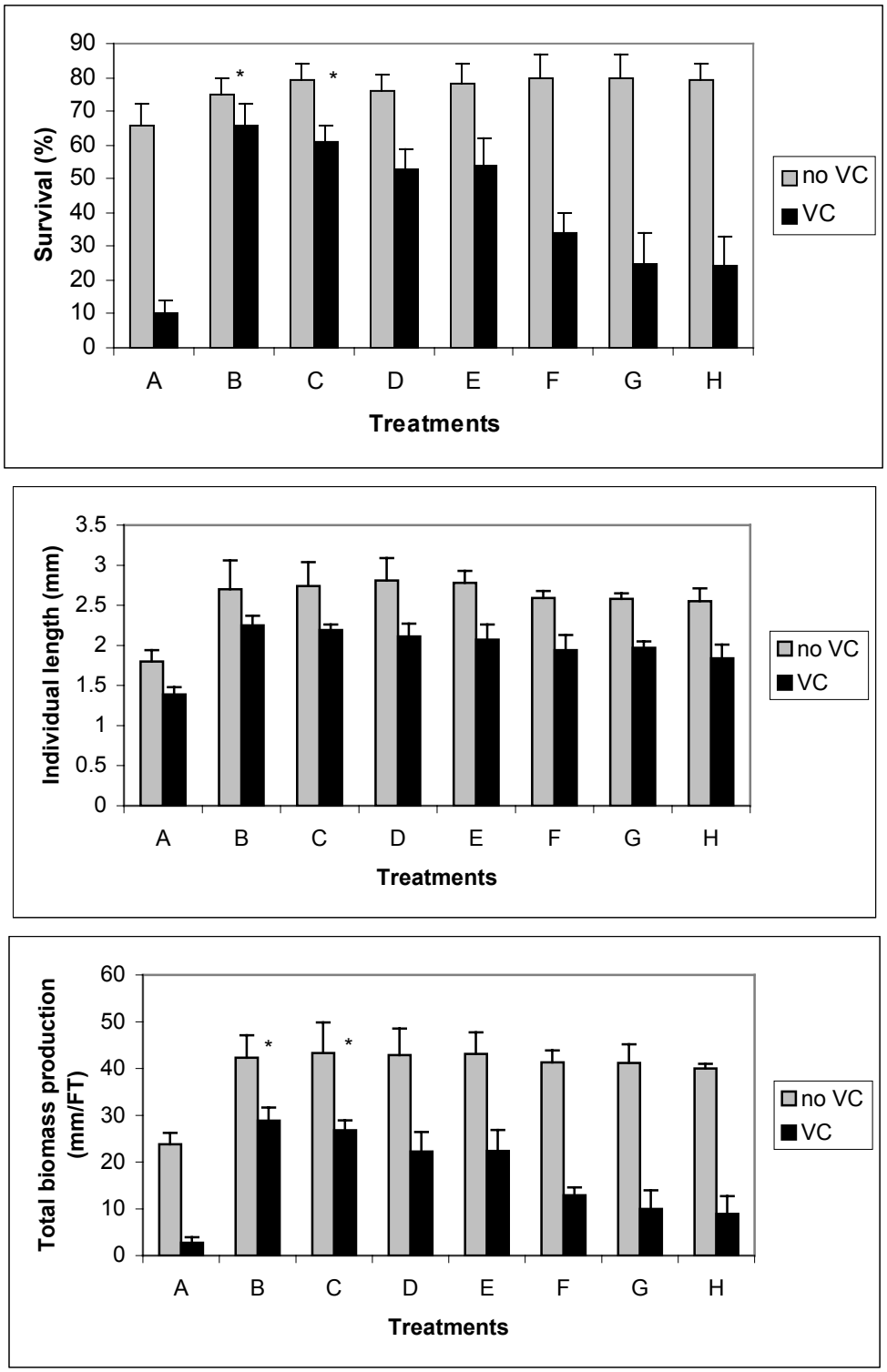


Fig. 5.4 Histogram of the average *Artemia* survival, individual length and total biomass production (mm/FT, and respective standard deviation) when fed various feed combinations on day 6. The mnn9 yeast cells (constituted 10% of the total AFDW) were supplied daily at different time intervals (either before ("-h") or after ("+h") challenge test) together with a combination of WT yeast and dead LVS3 (WT+LVS3). As a control, nauplii were not supplied with mnn9 yeast cells. A) corresponds to treatment: WT+dead LVS3. B) corresponds to treatment: WT+dead LVS3+ mnn9 10% (-16h). C) corresponds to treatment: WT+dead LVS3+ mnn9 10% (-8h). D) corresponds to treatment: WT+dead LVS3+ mnn9 10% (-4h). E) corresponds to treatment: WT+dead LVS3 mnn9 10% (-2h). F) corresponds to WT+dead LVS3 mnn9 10% (0h). G) corresponds to WT+dead LVS3 mnn9 10% (+2h). H) corresponds to WT+dead LVS3 mnn9 10% (+4h). Grey bars represent results of unchallenged nauplii while black bars reperesents nauplii challenged with *Vibrio campbellii* (VC) added on day 3. Asterisks mean no significant differences between two bars (p T*ukey*>0.05).

In experiment 4, the protective effect of short-term exposure (8h priming) of *Artemia* to WT and mnn9 yeast cells was studied (see Table 5.5). In the absence of a challenge, a short-term exposure to yeast cells had a strong beneficial effect on survival, lasting until day 3 (Table 5.5: treatment 4 and 6), while nauplii challenged immediately after the priming displayed some beneficial effect of the priming on day 2, irrespective of the genetic background of the yeast offered (Table 5.5: treatment 5 and 7). The priming effect was statistically stronger with mnn9 yeast than with WT cells (Table 5.5: treatment 7 versus treatment 5). On day 3, no nauplii survived in the LVS3 control treatment (Table 5.5: treatment 3) nor in the WT priming experiment, while in the mnn9 experiment a very small number of individuals survived.

Table 5.5 Experiment 4 - Mean daily survival (%) of *Artemia* fed with yeast cells (WT and mnn9) only for 8 h. The nauplii were subsequently challenged once with VC and fed with dead LVS3 at a density of 10^7 cells/ ml/day during 2 days. As a control, nauplii were only fed with dead LVS3 during 2 days. Each experiment was repeated twice (A and B). Each feed was tested in four replicates. Means were put together with the standard deviation (mean±SD.). Values in the same column showing the same superscript letter are not significantly different ($p_{Tukey}>0.05$). TN corresponds to treatment number.

Treatments		Priming (0-8h)	Feed (8-48h)	Challenge (8h)	Survival (%) A		Survival (%) B	
					Day 2	Day 3	Day 2	Day 3
1	No feed	-	-	-	26 ± 6^{d}	0^{c}	31 ± 5^{e}	0^{d}
2	LVS 3	-	LVS 3	-	86 ± 3^{a}	25 ± 4^{b}	81 ± 5^{b}	30 ± 4^{b}
3	LVS 3+live VC	-	LVS 3	VC	24 ± 3^{d}	0^{c}	20 ± 4^{f}	0^{d}
4	WT+LVS 3	WT	LVS 3	-	88 ± 3^{a}	41 ± 6^{b}	84 ± 3^{b}	38 ± 5^{b}
5	WT+LVS 3+live VC	WT	LVS 3	VC	56 ± 5^{c}	0^{c}	54 ± 5^{d}	0^{d}
6	mnn9+LVS 3	mnn9	LVS 3	-	90 ± 4^{a}	61 ± 10^{a}	94 ± 3^{a}	66 ± 5^{a}
7	mnn9+LVS 3+liveVC	mnn9	LVS 3	VC	75 ± 4^{b}	4 ± 3^{c}	70 ± 4^{c}	8 ± 3^{c}

4. Discussion

Data presented in the first experiment (Table 5.2), confirmed the results from Marques *et al*. (2006) that daily addition of small amounts of mnn9 yeast and/or pure glucan to a poor performing feed protects *Artemia* against the pathogenic VC while *Artemia* not supplemented with these compounds can not resist this pathogen. These data also suggest that glucan particles in an axenic environment do not have a nutritional value (see Table 5.2, line 1 vs. 5, survival and IL are not statistically different). Although the exact mechanism involved in such protection is not clear, β-glucans and

chitin (present in high concentrations in the mnn9 yeast cell wall) have been hypothesized to play an important role in the protection of *Artemia* against pathogenic bacteria. This hypothesis is corroborated by Vismara *et al.* (2004), who obtained a significant increase in *Artemia* resistance against stress conditions, by the daily administering to the *Artemia* nauplii of a *Euglena gracilis* mutant rich in β-glucans.

Although is not clear in *Artemia*, Cheng *et al*. (2005) demonstrated that β-glucans provided to other crustaceans, such as shrimp, seem to bind specific recognition proteins present in haemocytes. Circulating haemocytes play extremely important roles in the crustacean immune system (Smith *et al.,* 2003). Once β-glucans are detected, haemocytes are activated and attempt to neutralize or eliminate infective agents, by directly sequestrating and killing infectious agents through phagocytosis and encapsulation, or by releasing a battery of potent bioactive molecules that assist phagocytosis, such as microbicidal proteins (*e.g.* lectins), agglutinins, hydrolytic enzymes and antimicrobial peptides stored in these cells (Smith and Chisholm, 2001; Soderhall and Cerenius, 1998). In the mnn9 mutant, the yeast cell-wall composition is considerably changed *i.e* increased cell-wall bound chitin and glucans in combination with reduced mannoproteins (Marques *et al.,* 2004a). These changes increase yeast digestibility to *Artemia*, thus allowing nauplii to perform better in comparison to WT yeast (Marques *et al.,* 2004a). The second set of experiments investigated the dynamics of the induction of the protecting effect. In unchallenged nauplii offered mnn9 yeast (under various feeding periods) the TBP was significantly higher (mostly due to higher IL) in comparison to nauplii deprived of these yeast cells. Interestingly, there was no significant difference in TBP among these treatments although they were fed with mnn9 yeast for different periods. However, the nauplii, which received mnn9 yeast for at least 8 h before challenge, could withstand the pathogen. In these treatments, the performance of *Artemia* decreased gradually (due to both lower survival and lower IL) as they were exposed for a shorter time to mnn9 yeast cells. However, in all treatments (either challenged or not) mnn9 yeast generally improved nauplii IL. This suggests that mnn9 yeast cells when supplied even in small quantities seem to fulfill a double function, as they can boost IL of *Artemia* as well as protect them in a challenge test. In contrast to

mnn9, pure glucan was not able to boost the IL of *Artemia* nauplii in a significant way (as also observed in experiment 1) *i.e.* it could only protect *Artemia* against the pathogen when offered for at least 48h prior to challenge (since start feeding *Artemia* on day 1). Therefore, mnn9 yeast could induce protection in *Artemia* against the pathogen faster compared to pure glucan particles. Two possible mechanisms can be formulated to explain the observed differences. Possibly the β-glucans in mnn9 cells are more appropriate than pure β-glucan (*e.g.* the ratio of β-1,3 and β-1,6-glucan, the molecular weight, the dimensional structure, type and frequency of branches) (Ai *et al.,* 2007). Furthermore, apart from β-glucans, mnn9 yeast cells contain chitin, which has been proven to stimulate the immune system in crustaceans (Anderson and Siwicki, 1994; Song and Huang, 1999) and thus may work in synergism with β-glucans. In addition to its immunostimulatory nature, mnn9 cells might have a nutritional effect as well, which can stimulate/activate the digestive physiology (enzyme secretion) in the same way as has been described earlier in European sea bass *Dicentrarchus labrax* supplied with microalgae (Cahu *et al.,* 1998) or baker's yeast (Tovar *et al.*, 2002). In the present study neither mnn9 nor pure glucan showed any curative effect when supplied after challenge.

In the final set of experiments, we tested whether exposing *Artemia* for a short-term (8h priming) to WT or mnn9 yeast cells would be sufficient for their protection against pathogenic bacteria. After priming, the nauplii only received dead bacteria (LVS3) as feed, which is known not to protect *Artemia* against *V. campbellii*. Although the priming effect was significantly stronger with mnn9 yeast than with the WT cell, this effect was short-lived and disappeared very quickly one day after challenge. To date, no evidence for adaptive immune system has been found in invertebrate or lower vertebrates (lampreys and hagfish), although there is some evidence in invertebrates, that priming the innate immune system can have a medium to long-term protective effect (Little and Kraaijeveld, 2004). Previous priming studies (Moret and Siva-Jothy, 2003) with the mealworm beetle, *Tenebrio molitor*, revealed a long-term protection (50 days) against the pathogenic fungus *Metarhizium anisopliae* after priming the innate immune system with lipopolysaccharides (LPS). Although the compound used in the present study is different, both LPS and glucans are

immunostimulants for which the innate immune system has specific receptors (Janeway and Medzhitov, 2002), resulting in a general antimicrobial response (Beutler, 2004). The actual mechanism that maintains high level of antimicrobial activity during long-lasting immune responses in invertebrates (*e.g.* insects) is as yet not known and such a persistent antimicrobial activity may either be due to a continuous synthesis of antimicrobial peptides in the haemolymph or to a slow **t**urnover of these peptides when produced (Moret and Siva-Jothy, 2003). According to Vierstraete (2004) the type of priming agent is very important to trigger the immune system in invertebrates. They identified different protein profiles in haemolymph of *Drosophila* 25 min after priming with *Micrococcus luteus*, *Saccharomyces cerevisiae* and lipopolysaccharides (LPS), suggesting that the interaction between invertebrates (and also vertebrate hosts) and their pathogens may be extremely specific (Kurtz and Franz, 2003; Little *et al.,* 2003; Decaestecker *et al.,* 2003; Witteveldt *et al.,* 2004). Hence, the priming of the innate immune system of invertebrates could depend on several factors such as the specificity and quantity of a compound used as well as the animal tested. Biochemical data will be needed to provide evidence that the increased β-glucan and/or chitin content of the mnn9 strain is responsible for this short-lived priming effect.

Summarizing, mnn9 yeast and pure glucan could provide protection in *Artemia* against *Vibrio campbelli* when supplied well ahead of the challenge. The protection by mnn9 is short-lived when they are used to prime the immune system. The mechanism involved in such protection is not yet clear but two hypotheses can be put forward: if *Artemia* nauplii which may hatch with a sufficient amount of biochemically efficient haemocytes to fight diseases, one may expect an immediate response by the immunostimulants and even priming could be sufficient to overcome diseases. Alternatively, it is possible that *Artemia* nauplii may hatch without haemocytes and consequently with an under developed immune-e system. Unfortunately, we know very little about the ontogeny of the immune system in invertebrates (in *Artemia*, especially) as in general their small size precludes direct study. Published work to date has focused on the development of the immune system in only bivalve molluscs and echinoderms (Dyrynda *et al.,* 1995). It cannot be assumed that juveniles exhibit the same responses as adults, or that the expression of immune proteins occurs to

the same degree. To gain insight into the immune capability of penaeid shrimp during larval development, in a period of life when shrimp are particularly susceptible to infection, the expression and localization of penaeidin (Litvan-Pen3-1) have been studied in the first larval stages, from nauplius V, Zoea I, II, III, to Mysis II, and in post-larvae (Munoz *et al.,* 2003). The results showed Litvan-Pen3-1 transcript and peptides are restricted to some haemocytes observed in the Mysis II larval stage. This suggests either a low level of transcriptional activity in expressing cells, or a low number of expressing cells present in the larvae (Munoz *et al.,* 2003).

In the marine mussel, *Mytilus edulis,* the levels of phagocytosis in immature larval haemocytes (*e.g.* in trochophore and veliger cells) was much lower (in both percentage of phagocytic cells and mean number of bacteria ingested per cell) in respect to adult mussel haemocytes (Munoz *et al.,* 2003).

Considering these limited literature data, our results are in more agreement with the second hypothesis, which postulates that *Artemia* nauplii are hatching with an insufficient amount of haemocytes or with a poorly functional immune system. Consequently, traces of glucan and mnn9 yeast are likely triggering the proliferation or the maturation of the haemocytes, justifying the time lag between the addition of the immunostimulants and the appearance of the protective effect. However, the immune induction obtained by these products in *Artemia* nauplii may not be necessarily similar in more advanced developmental stages such as juvenile or adult *Artemia*. Further studies on the relationship between gene expression and survival of challenged nauplii in gnotobiotically-grown *Artemia* could elucidate in detail the mechanism of action of yeast cells and/or specific compounds on the innate immune system.

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CHAPTER VI

Enhanced disease resistance in *Artemia* by application of commercial β-glucans sources and chitin in a gnotobiotic *Artemia* challenge test

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Abstract

The anti-infectious potential of a selection of putative immunostimulants including six commercial β-glucans (all extracted from baker's yeast *Saccharomyces cerevisiae* except for Laminarin) and chitin particles were verified in *Artemia* nauplii by challenging them under gnotobiotic conditions with the pathogen *Vibrio campbellii*. Under the described experimental conditions, no differential macroscopic nutritional effect (*e.g.* growth) was observed among the products. Significant increased survival was observed with β-glucan (Sigma) and Zymosan and to a lesser extent with MacroGard in challenged nauplii. A poor correlation was found between survival values of the challenged *Artemia* and the product compositions (such as chitin, mannose and β-glucan content) indicating that the quality of β-glucans (*e.g.* the ratio of β-1,3 and β-1,6-glucan, the molecular weight, the dimensional structure, type and frequency of branches), eventually in combination with other unidentified compounds, is more important than the amount of product offered. This small-scale testing under gnotobiotic conditions using freshly-hatched *Artemia* nauplii allows for a rapid and simultaneous screening of anti-infectious and/or putative immunostimulatory polymers, and should be combined with studies on cellular and humoral immune responses in order to gain more quantitative insight into their functional properties.

1. Introduction

Diseases are still a major constraint to sustainable aquaculture production, especially for the farming of invertebrates (Bachére, 2003). According to Raa (2000), invertebrates are apparently entirely dependent on non-specific immune mechanisms to cope with infections, as they lack the specific immunological "memory" that is found in fish and warm-blooded animals. As a result, it does not seem to make sense to vaccinate them against specific diseases. Nowadays, the use of preventive and environment-friendly

approaches such as probiotics, immunostimulants, antibacterial peptides and quorum-sensing systems are becoming increasingly important in aquaculture (Bachére, 2003; Sakai, 1999; Verschuere *et al.,* 2000; Defoirdt *et al.,* 2005). However, the application of such technologies must be based on a thorough understanding of the mechanisms involved and the putative consequences. An essential part of that understanding can be provided by studies of host-microbial interactions. A key experimental approach to study these interactions is to first define the functioning of the host in the absence of bacteria and then to evaluate the effect of adding a single or defined population of microbes, or certain compounds *i.e.* under axenic or gnotobiotic conditions (Gordon and Pesti, 1971 ; Marques *et al.,* 2004a,b). Marques *et al*. (2004a) have recently developed and validated the usefulness of an *Artemia* gnotobiotic test system allowing to study the nutritional effect of food composition as well as the host-microbial interaction.

The use of specific biological compounds (immunostimulants) that enhance immune responses of target organisms, rendering animals more resistant to diseases may be an excellent preventive tool against pathogens. Several immunostimulants have been used in vertebrate and invertebrate culture, to induce protection against a wide range of diseases: *i.e.* β-glucans (Sung *et al.,* 1996; Sritunyalucksana *et al.,* 1999; Burgents *et al.,* 2004; Misra *et al.,* 2004), chitin (Anderson and Siwicki, 1994; Song and Huang, 1999; Wang and Chen, 2005), mannoproteins (Tizard *et al.,* 1989), lipopolysaccharides (Takahashi *et al.,* 2000), peptidoglycans (Itami *et al.,* 1998; Boonyaratpalin *et al.,* 1995) and dead bacteria (Alabi *et al.,* 1999; Keith *et al.,* 1992; Vici *et al.,* 2000).

Nevertheless, rigorous analysis of the results obtained in most experiments reveals that the validity of some conclusions with respect to the benefit of immunostimulation is limited, due to mainly poor experimental design, to the absence of any statistical analysis and to poor reproducibility of the results (Smith *et al.,* 2003). Therefore, Smith *et al.* (2003) argued that there is an urgent need to provide unequivocal evidences of the beneficial effects of immunostimulants using standardized trials under controlled rearing conditions, complemented with fundamental research on defence mechanisms. These trials could be performed with gnotobiotically-cultured invertebrates (animals cultured in a totally controlled microbial environment).

The present study aims to verify the putative pathogen-protective effect of some commercial β-glucans and chitin particles in a gnotobiotic *Artemia* challenge test system using the opportunistic pathogenic bacterium *Vibrio campbellii.*

2. Methodology

2.1. Bacterial strains and growth conditions

Two bacterial strains were used, *i.e. Aeromonas hydrophila* strain LVS3 for its positive effect on *Artemia* performance when fed at sub-optimal concentration (Soto-Rodriguez *et al.,* 2003; Gomez-Gil *et al.,* 2004; Marques *et al.,* 2005) and *Vibrio campbellii* strain LMG21363 (VC) for its pathogenic effect towards *Artemia* and shrimp (Marques *et al.,* 2005; Soto-Rodriguez *et al.,* 2003; Gomez-Gil *et al.,* 2004; Coutteau *et al.,* 1990). The two bacterial strains were cultured and harvested according to procedures described by Marques *et al.* (2005). Bacteria were resuspended in filtered and autoclaved seawater (FASW, 0.2 µm) and their densities determined by spectrophotometry (OD_{550}), assuming that an optical density of 1.000 corresponds to 1.2×10^9 cells/ml, according to the McFarland standard (Biomerieux, Marcy l'Etoile, France). Dead LVS3 was fed to *Artemia* using aliquots of autoclaved bacteria (autoclaving at 120 °C for 20 min). At day 3, challenge tests were performed with live VC according to a procedure described by Soltanian *et al.* (2007).

The composition of the products was determined using high performance anionic exchange chromatography (HPAEC) (Dionex Bio-LC50 system, Sunnyvalle, USA), according to the methodologies described by Dallies *et al.* (1998).

2.2. Artemia gnotobiotic culture

Experiments were performed with *Artemia franciscana* cysts, originating from Great Salt Lake, Utah-USA (EG ® type, INVE Aquaculture SA, Dendermonde, Belgium). All manipulation was carried out under a lamina flow hood and all necessary tools were previously autoclaved at 120 °C for 20 min. Bacteria–free cysts and nauplii were obtained using the procedure described by Sorgeloos *et al.* (1986) and Marques *et al.* (2004a). After hatching, 20 axenic nauplii (Instar II) were transferred to Falcon tubes

containing 30 ml of FASW together with the amount of feed scheduled for day 1. The daily feeding schedule was adopted from Soltanian *et al*. (2007) and is intended to provide *ad libitum* ratios but avoiding excessive feeding in order not to affect the water quality in the test tubes. In all experiments the total amount of dead LVS3 provided to *Artemia* was approximately 10.5×10^9 cells/FT (distributed in 5 daily feeding portions; daily fraction (in %) 9:17:17:23:34). Each treatment consisted of four Falcon tubes (replicates), placed on a rotating rod at 4 cycles per min and exposed to constant incandescent light ($\pm$ 41µEm^{-2}) at 28°C. Tubes were being transferred to the laminar flow just once per day for feeding.

2.3. Method used to verify axenity

Axenity of feed, decapsulated cysts and *Artemia* cultures was checked at the end of each experiment using a combination of plating (Marine Agar) and live counting (using tetrazolium salt MTT (-3-(4,5-dimethylthazol-2, 5-diphenyl tetrazolium bromide) (Sigma, 0.5 % w/v)) staining following the procedure described by Marques *et al.* (2006). In challenge treatments, the axenity of *Artemia* culture was always checked before challenge using the same methods. Contaminated culture tubes were not considered for further analysis and the treatment was repeated.

2.4 Particles of glucans and chitin

Insoluble particles of chitin (Sigma, from crab shell, 1g) and five commercial β-glucans, namely, Biorigin (Betamune, Brazil), MacroGard (MacroGard®, Biotec-Mackzymal, Norway), Immunowall (Brazil), Zymosan (Sigma, 1g) and the β-glucan (Sigma, 100mg) (all obtained from baker's yeast *Saccharomyces cerevisiae)* in addition to a soluble β-glucan (Laminarin, Sigma, 500 mg) extracted from *Laminaria digitata*, were tested in the *Artemia* gnotobiotic challenge test system to verify their potential to protect *Artemia* nauplii against the pathogenic VC. Non-sterile compounds (Biorigin, Immunowall and MacroGard) were suspended in absolute ethanol (100%) in sterile 50 ml Falcon tubes with loose cap and put at 28° to dry. Particles were aseptically transferred to sterile 50 ml Falcon tubes and homogeneously suspended in FASW. Contaminations were checked by plating the suspension on MA (100µl, n=2). Absence of bacterial growth was monitored after incubating plates for 5 days at 28°C. No bacterial

growth was detected on marine agar after 5 days of incubation. An optical laser particle size analyser (Mastersizer MSX-17, Malvern Instruments Ltd., Malvern, Works, UK; resolution 0.05-900 μm) was used to determine the diameter of particles present in the suspension, using the software Malvern Mastersizer S version 2.19®. Each product had its specific particle distribution curve. The volume percentage of particles, sizing less than 50μm (maximum particle size that can be ingested by *Artemia*) was calculated (Table 6.1, second column). In a classical six day experiment (Marques *et al*., 2004a), 1.06 mg AFDW (ash free dry weight) of yeast cells are offered to *Artemia* per tube. According to Marques *et al*. (2004a), this corresponds on average, depending on the yeast strains, to 213 μg of cell wall material. Here it was decided, on an arbitrary basis, to add 128 μg of ingestible product (being 60%). In order to provide equal amounts of ingestible particles it was necessary to adjust the feeding regime for each product (see legend Table 6.1). The percentage of ingestible (lower than 50 μ) particles in combination with total amount of β-glucans offered to *Artemia* is presented in Table 6.1.

Table 6.1 Particle diameter distribution of commercial glucans and chitin offered to *Artemia* nauplii in experiment 1 and 2. Only the particles with the size less than 50µm are considered to be ingestible by *Artemia* nauplii. The amount of particles offered to *Artemia* was calculated as 128 µg/5 feedings/% ingestible particles/100. The total amount of ingestible glucan offered was calculated as the amount of particles offered × % sugar content/100 × % glucan content/100. (For sugar content and glucan content, see Table 1, column 2 and 5 respectively).

Product	Percentage (%) of particle size less than 50 µm	Particles offered (µg/tube/day)	Total Dry Weight (DW) of ingestible glucan offered (µg/tube/day)
Zymosan	100	26	15.7
β-glucan (Sigma)	12.11	214	13.1
Biorigin	18	145	18.2
MacroGard	74	35	11.8
Immunowall	100	26	5.3
Chitin	60	21	-
Laminarin	soluble	26	21.7

Experiments in which 3 times as much particles of the respective products were offered, were also performed. The suspension of particles was stored at 4°C until the end of the experiment. It was verified if the particles in the different commercial preparations could display a differential adhesion of *Vibrio* cells, potentially influencing the number of pathogenic bacterium (VC) ingested by *Artemia* in the challenge test. However, no bacterial adherence to particles was observed when checked under the microscope (1000x) over a period of 48h of contact between them (results not shown).

2.5. Experimental design

This study comprises 2 experiments and their experimental design is schematised in Fig. 6.1. In experiment 1 and 2, *Artemia* nauplii were fed daily with poor-performing and non-protective dead LVS3 (a total 10.5×10^9 cells/Falcon Tube (FT) for 6 days) as a major feed source in combination with small amount of glucans and chitin particles (approximately 2% of total amount of feed offered). Glucan and chitin additions were divided in equal parts per day, to avoid the possibility that those particles could be used as major feed source by *Artemia*) (Fig. 6.1).

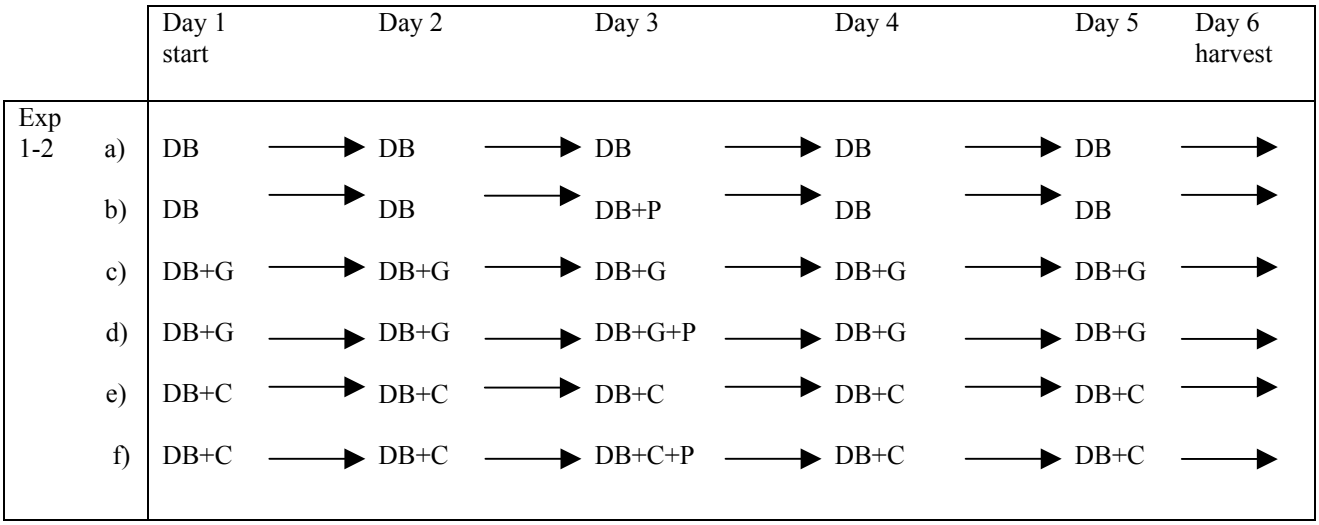

Fig. 6.1 Experimental design of two experiments (Exp) performed. Legend: a)-f) correspond to the treatmentsperformed; DB – dead bacteria LVS3; P – pathogen (*Vibrio campbellii*); G – glucans (Zymosan, β-glucan (Sigma), Laminarin, Biorigin, MacroGard and Immunowall); C – chitin particles.

At day 3, challenge tests were performed with live VC. For that purpose, in a laminar flow hood, the pathogen was added to each replicate at a density of 5×10^6 cells/ml. As a control *Artemia* was only fed with the dead LVS3 and challenged (or not) with the pathogen. These experiments were repeated to verify the reproducibility of the results.

2.6. Survival and growth of Artemia

The survival percentage was determined daily for each treatment. For this purpose, the number of live *Artemia* was registered before feeding or adding bacteria by counting with the naked eye while exposing each transparent Falcon tube to an incandescent light without opening the tube to maintain the gnotobiotic environment. At the end of each experiment (day 6 after hatching), live *Artemia* were fixed with lugol's solution to measure their individual length (IL), using a dissecting microscope equipped with a drawing mirror, a digital plan measure and the software Artemia 1.0® (courtesy Marnix Van Damme). As a

criterion that combines both the effects of survival and IL, the total biomass production (TBP) was determined according to the following equation:

TBP (millimeters per Falcon tube - mm/FT) = number of survivors × mean IL

Relative percentage survival (RPS) value was determined in *Artemia* fed with every product or not (control) according to the following equation:

RPS (%) = (Percentage of surviving challenged *Artemia*)/(Percentage of surviving unchallenged *Artemia*) ×100

Regression analysis was carried out between *Artemia* survival (arcsine transformed values) and polymer concentration (chitin, mannose and β-glucans) in each product in order to test for possible correlation between these parameters.

2.7. Statistics

Values of larval survival (percentage) were arcsin transformed, while values of IL and TBP were logarithmic or square root transformed to satisfy normal distribution and homoscedasticity requirements. Differences in survival, RPS, IL and TBP of *Artemia* cultured in different conditions were investigated with analysis of variances (ANOVA) and Tukey's multiple comparison range. All statistical analyses were tested at the 0.05 level of probability, using the software SPSS 11.5 for Windows.

3. Results

Polymer composition of the commercial glucans used in this study is shown in Table 6.2. The composition analysis of the products indicates that the percentage of each polymers (chitin, mannose and β-glucans) varies between the products. Except for Immunonowall, all components contain a high proportion of β-glucan in their sugar fraction. The purity of the compounds (*e.g.* % of sugar) is however very variable.

Table 6.2 Differential polymer distribution (chitin, mannose and β-glucan) of six commercial glucans tested in the gnotobiotic *Artemia* challenge test.

		Percent (%) of polymer contribution			
Product	Sugar (%) dry weight	Chitin	Mannose	β-glucan	% glucan on dry weight
Zymosan	71.5	1.7	14.0	84.3	60.2
Laminarin	91.4	0	0.8	91.2	83.3
β-glucan (Sigma)	54.8	2.9	5.5	91.6	50.2
Biorigin	72.7	1.4	2.6	96.0	69.8
MacroGard	50.0	2.0	7.0	91.0	45.5
Immunowall	37.0	1.6	43.4	55.0	20.3

The effect of β-glucans and chitin particles on the survival of nauplii fed with dead LVS3 was tested in a challenge test with VC. The results were presented in Table 6.3 and 6.4 (Exp 1 and 2). No significant difference was observed in *Artemia* survival until day 3 (before challenge test) (data not shown). The results indicate that some of the challenged nauplii fed only with dead LVS3 (control treatment) could survive until day 6 although in general the performance was low (low survival, low RPS and low TBP). The addition of some products (Zymosan, β-glucan (Sigma) and MacroGard) was able to significantly improve the TBP in challenged nauplii (in comparison to challenged nauplii which did not receive these compounds), mainly due to higher *Artemia* survival values (Table 6.3 and 6.4, treatments: 2, 8 and 14 vs 18). On the other hand, the TBP of *Artemia* was always significantly reduced by challenging the nauplii, irrespective of the treatment. Not a single compound had a significant effect on *Artemia* growth (IL) under the described experimental conditions, as illustrated by identical average individual length values over all treatments in the absence of challenge. Moreover, application of higher concentrations of these products (up to three times) did not change the obtained challenge data (data not shown).

Table 6.3 Experiment 1- Mean daily survival (%), relative percentage survival (%) (RPS), individual length (IL) (mm) and total biomass production (TBP) (mm per Falcon Tube – FT) of *Artemia* fed daily with dead LVS3 alone or in combination with different types of glucan and chitin particles after 5 days. The challenge test was performed with *Vibrio campbelii* (VC) added on day 3 (D3). The first column in the table refers to the type of treatments. (see Fig. 6.1). TN means treatment number. Each feed was tested in four replicates. Values are presented with the respective standard deviation (mean ± SD). Values in the same column showing the same superscript letter are not significantly different ($p_{Tukey}>0.05$).

Experiment 1		Survival (%)					
TN		Day 4	Day 5	Day 6	RPS (%)	IL (mm)	TBP (mm/FT)
1	dead LVS3+Zymosan	83 ± 3^{abc}	79 ± 5^{abc}	75 ± 4^{abc}	82 ± 5^{a}	1.6 ± 0.1^{a}	23.5 ± 1.5^{ab}
2	dead LVS3+Zymosan+VC (D3)	78 ± 3^{cde}	70 ± 4^{bcd}	61 ± 2^{c}		1.5 ± 0.1^{a}	19.4 ± 0.8^{c}
3	dead LVS3+Laminarin	84 ± 5^{abc}	81 ± 3^{ab}	79 ± 3^{ab}	22 ± 7^{cd}	1.5 ± 0.1^{a}	24 ± 0.8^{a}
4	dead LVS3+Laminarin+VC D3)	64 ± 5^{f}	21 ± 8^{g}	18 ± 5^{f}		1.4 ± 0.1^{a}	3.5 ± 1.4^{g}
5	dead LVS3+Chitin	89 ± 3^{a}	86 ± 3^{a}	83 ± 3^{a}	10 ± 5^{d}	1.5 ± 0.1^{a}	20.3 ± 1.5^{bcd}
6	dead LVS3+Chitin+VC (D3)	63 ± 3^{f}	20 ± 7^{g}	9 ± 5^{f}		1.4 ± 0.1^{a}	2.5 ± 1.4^{g}
7	dead LVS3+Glucan	86 ± 3^{ab}	79 ± 5^{abc}	78 ± 3^{abc}	84 ± 9^{a}	1.6 ± 0.1^{a}	23.1 ± 0.8^{ab}
8	dead LVS3+Glucan+VC (D3)	79 ± 5^{bcde}	69 ± 5^{cd}	65 ± 7^{bc}		1.4 ± 0.1^{a}	18.7 ± 0.8^{c}
9	dead LVS3+Glucan+Chitin	84 ± 3^{abc}	80 ± 4^{abc}	75 ± 4^{abc}	88 ± 3^{a}	1.6 ± 0.1^{a}	23.6 ± 1.3^{a}
10	dead LVS3+Glucan+Chitin+VC (D3)	80 ± 4^{bcd}	70 ± 4^{bcd}	66 ± 5^{c}		1.5 ± 0.1^{a}	20.3 ± 1.5^{bc}
11	dead LVS3+Biorigin	84 ± 3^{abc}	78 ± 3^{abc}	70 ± 4^{bc}	23 ± 9^{cd}	1.5 ± 0.1^{a}	21.4 ± 1.2^{abcd}
12	dead LVS3+Biorigin+VC (D3)	70 ± 4^{ef}	36 ± 8^{ef}	16 ± 6^{ef}		1.4 ± 0.1^{a}	4.6 ± 1.8^{fg}
13	dead LVS3+MacroGard	83 ± 3^{abc}	78 ± 3^{abc}	74 ± 3^{abc}	63 ± 3^{b}	1.6 ± 0.1^{a}	23.1 ± 0.8^{ab}
14	dead LVS3+MacroGard+VC (D3)	71 ± 3^{def}	56 ± 3^{d}	46 ± 3^{d}		1.4 ± 0.1^{a}	13.3 ± 0.7^{e}
15	dead LVS3+Immunowall	83 ± 3^{abc}	76 ± 3^{abc}	70 ± 4^{bc}	36 ± 12^{c}	1.5 ± 0.1^{a}	21.2 ± 1.2^{abcd}
16	dead LVS3+Immunowall+VC (D3)	65 ± 4^{f}	41 ± 6^{e}	25 ± 7^{e}		1.4 ± 0.1^{a}	7.0 ± 2.0^{f}
17	dead LVS3	83 ± 3^{abc}	78 ± 3^{abc}	70 ± 6^{bc}	22 ± 7^{cd}	1.4 ± 0.1^{a}	19.4 ± 1.6^{d}
18	dead LVS3+VC (D3)	68 ± 3^{f}	28 ± 6^{fg}	15 ± 4^{ef}		1.4 ± 0.1^{a}	4.1 ± 1.1^{fg}

Table 6.4 Experiment 2- Mean daily survival (%), relative percentage survival (%) (RPS), individual length (IL) (mm) and total biomass production (TBP) (mm per Falcon Tube – FT) of *Artemia* fed daily with dead LVS3 alone or in combination with different types of glucan and chitin particles after 5 days. The challenge test was performed with *Vibrio campbellii* (VC) added on day 3 (D3). The first column in the table refers to the type of treatments. (see Fig. 6.1). TN means treatment number. Each feed was tested in four replicates. Values are presented with the respective standard deviation (mean ± SD). Values in the same column showing the same superscript letter are not significantly different ($p_{Tukey}>0.05$).

Experiment 2	Survival (%)					
TN	Day 4	Day 5	Day 6	RPS (%)	IL (mm)	TBP (mm/FT)
1 dead LVS3+Zymosan	85 ± 4^{abc}	79 ± 3^{ab}	74 ± 5^{abc}	86 ± 3^{a}	1.5 ± 0.1^{ab}	22.1 ± 1.2^{a}
2 dead LVS3+Zymosan+VC (D3)	79 ± 3^{abcd}	69 ± 8^{bc}	64 ± 2^{cd}		1.4 ± 0.1^{ab}	17.6 ± 2.5^{bc}
3 dead LVS3+Laminarin	83 ± 3^{abc}	81 ± 3^{a}	79 ± 3^{a}	16 ± 5^{cd}	1.5 ± 0.1^{ab}	23.2 ± 0.7^{a}
4 dead LVS3+Laminarin+VC (D3)	61 ± 3^{g}	29 ± 3^{ef}	13 ± 5^{f}		1.3 ± 0.1^{ab}	4.3 ± 1.3^{de}
5 dead LVS3+Chitin	79 ± 5^{abcd}	75 ± 4^{ab}	73 ± 3^{abc}	19 ± 10^{cd}	1.5 ± 0.1^{ab}	22 ± 0.9^{a}
6 dead LVS3+Chitin+VC (D3)	66 ± 5^{efg}	21 ± 8^{f}	16 ± 8^{f}		1.4 ± 0.1^{ab}	3.8 ± 2.1^{e}
7 dead LVS3+Glucan	83 ± 3^{abc}	80 ± 4^{ab}	74 ± 3^{abc}	84 ± 10^{a}	1.5 ± 0.1^{a}	21.9 ± 0.8^{a}
8 dead LVS3+Glucan+VC (D3)	76 ± 3^{bcde}	71 ± 3^{abc}	65 ± 7^{cd}		1.4 ± 0.1^{ab}	16.6 ± 2.3^{c}
9 dead LVS3+Glucan+Chitin	80 ± 4^{abcd}	80 ± 4^{ab}	74 ± 6	92 ± 6^{a}	1.5 ± 0.1^{ab}	22.7 ± 1.2^{a}
10 dead LVS3+Glucan+Chitin+VC (D3)	75 ± 4^{cde}	69 ± 5^{bc}	67 ± 3^{bcd}		1.3 ± 0.1^{ab}	16.8 ± 1.7^{bc}
11 dead LVS3+Biorigin	85 ± 4^{ab}	78 ± 3^{ab}	71 ± 3^{abc}	28 ± 9^{cd}	1.5 ± 0.1^{a}	21.6 ± 0.8^{a}
12 dead LVS3+Biorigin+VC (D3)	73 ± 3^{cdef}	40 ± 7^{de}	20 ± 7^{ef}		1.4 ± 0.1^{ab}	5.5 ± 2^{de}
13 dead LVS3+MacroGard	83 ± 3^{abc}	74 ± 3^{ab}	71 ± 3^{abc}	68 ± 8^{b}	1.5 ± 0.1^{ab}	21.7 ± 0.8^{a}
14 dead LVS3+MacroGard+VC (D3)	73 ± 3^{cdef}	61 ± 3^{c}	49 ± 5^{d}		1.5 ± 0.1^{ab}	14.2 ± 1.4^{c}
15 dead LVS3+Immunowall	81 ± 5^{abcd}	71 ± 5^{abc}	69 ± 3^{abc}	41 ± 6^{c}	1.5 ± 0.1^{ab}	20.4 ± 0.7^{ab}
16 dead LVS3+Immunowall+VC (D3)	71 ± 3^{cdefg}	43 ± 6^{d}	26 ± 5^{e}		1.3 ± 0.1^{ab}	7.6 ± 1.3^{d}
17 dead LVS3	86 ± 3^{a}	79 ± 3^{ab}	74 ± 3^{ab}	24 ± 4^{cd}	1.4 ± 0.1^{ab}	20.9 ± 1.1^{a}
18 dead LVS3+VC (D3)	63 ± 3^{fg}	28 ± 3^{f}	18 ± 3^{ef}		1.3 ± 0.1^{b}	4.5 ± 0.7^{de}

4. Discussion

β-Glucans have been successfully used to enhance resistance of crustaceans against bacterial and viral infections (Chang *et al.,* 1999; Itami *et al.,* 1994; Kou and Song, 1994; Su *et al.,* 1995; Liao *et al.,* 1996; Song *et al.,* 1997; Chang *et al.,* 2000). The present study confirms the results of Marques *et al.* (2006). That study has shown that a daily addition of small amounts of β-glucan (Sigma) to *Artemia,* fed with a poor performing feed, enhanced resistance of this organism against the pathogenic VC, while *Artemia* solely fed with dead LVS3 cells could not resist this pathogen. Here, the addition of β-glucan (Sigma) was able to significantly improve *Artemia* TBP and RPS challenged with VC (mostly due to improvement in survival) compared to the nauplii which did not receive that glucan and were challenged with this pathogen (Tables 6.3 and 6.4; line 8 vs 18). The administration of different forms of β-glucan in the diet of different shrimp species has been shown to result in an enhancement of protection against various pathogens (Itami *et al.,* 1994; Kou and Song, 1994; Su *et al.,* 1995; Liao *et al.,* 1996; Song *et al.,* 1997; Chang *et al.,* 2000, 1996; Hennig *et al.,* 1998; Johansson, 2000). This increased resistance has been attributed to the enhancement of phagocytic activity of haemocytes (Itami *et al.,* 1994). Nevertheless, in some cases no beneficial effects were observed by using β-glucans. It was reported that survival of juvenile *Litopenaeus vannamei* fed a glucan-supplemented diet was even less than the control animals over seven-week periods (Scholz *et al.,* 1999).

Bath administration of glucan has been proven to be a suitable procedure to enhance the immune response and disease resistance in shrimp (Sung *et al.,* 1996; Ravichandran *et al.,* 2005; Chang *et al.,* 2003; Misra *et al.,* 2004). However, such enhancement varies with dose and type of glucan, feeding regime, test animal (Ai *et al.,* 2007) and developmental stage of the target organism. Furthermore, the biological effects of immunostimulants are highly dependent on the specificity of the receptors on the target cells recognising them as potential high-risk molecules and triggering defence pathways

(Bricknell and Dalmo, 2005). Some authors mentioned that a high level of β-1,3-glucan directly induced the respiratory burst, which, after a period, can exhaust the immune cells resulting in immunosuppression or feedback regulation (Misra *et al.,* 2004; Ai *et al.,* 2007; Castro *et al.,* 1999; Robertsen *et al.,* 1994).

Therefore, in the present study only small amounts of putative immune-enhancers were applied to *Artemia* likely not to over-stimulate the immune system. However, when the concentration of the applied β-glucans was increased (up to 3 fold) no changes were observed in the results.

Zymosan is one of the commercial β-glucans used in this study. It was described as a crude yeast cell-wall preparation of *S. cerevisiae* containing a relatively crude mixture of proteins, lipids and polysaccharides that was able to stimulate non-specific immunity (Fitzpatrick and DiCarlo, 1964; Freimund *et al.,* 2003). We obtained a significant increased resistance against VC (high RPS value) when *Artemia* nauplii were supplemented with small amounts of Zymosan and challenged with this pathogen (Table 6.3 and 6.4; lines; 2 vs 18). Actually, the results are very similar to the results obtained by using β-glucan (Sigma) in this study. Although Zymosan is not a very pure β-glucan (only 84% of the sugars are made up of β-glucan) (see Table 6.2), apparently the level and the type of β-glucan it contains is appropriate to up regulate the immune system of *Artemia* as does β-glucan (Sigma). In crayfish, Zymosan can activate the prophenoloxidase (pro PO) system, which is considered an important component in the innate defence of arthropods (Cardenas *et al.,* 1997). Sung *et al.* (1996) showed that Zymosan treatment in shrimp via immersion significantly increased anti-*E. coli* activity of plasma, as well as superoxide anion (O_2^-) and PO activity of shrimp haemocytes. These enhanced microbicidal reactions increased the clearance ability of haemolymph against the invasive pathogen *Vibrio vulnificus* (Sung *et al.,* 1996).

MacroGard, a cell wall extract from *Saccharomyces cerevisiae,* is another type of β-glucan tested in the gnotobiotic challenge test system. Administration of MacroGard by immersion has been shown to cause a transient increase in phenoloxidase enzyme activity and superoxide production (O_2^-) in

P. monodon (Sung *et al.,* 1996). Similarly, immersion of shrimp post-larvae in a suspension of MacroGard enhanced growth performance, immune response, and disease resistance in black tiger shrimp (Supamattaya *et al.,* 2000). In the present study, MacroGard improved *Artemia* survival, RPS and TBP, providing some level of pathogen resistance when challenged with VC (Table 6.3 and 6.4, line 14 vs 18), although the results, for unknown reasons, were not as spectacular as with β-glucan (Sigma) or Zymosan.

Laminarin (a water-soluble β-1,3-glucan derived from the brown algae *Laminaria digitata)*, was the other type of commercial β-glucan tested in the present study. Although some investigations documented positive immunostimulatory effects of Laminarin, no beneficial effect was observed in this study (low RPS and low TBP in challenged nauplii) (Table 3 and 4; lines; 4 vs 18). The effects of Laminarin on the haemocytes of the freshwater crayfish, *Astacus astacus*, and the shore crab, *Carcinus maenas*, were studied *in vitro* and *in vivo* to determine the role of the PO activation system, in the cellular defense reactions of crustaceans (Smith and Söderhäll, 1983). *In vitro*, phagocytosis of the bacterium, *Moraxella* sp. was significantly raised by addition of Laminarin. *In vivo*, injection of Laminaran (0.2 mg /ml haemolymph) into the hemocoel of *A. astacus* or *C. maenas* caused a rapid, marked reduction in the number of circulating haemocytes, indicating that a cellular defense reaction was initiated (Smith and Söderhäll, 1983). So although Laminarin seems to have a proven *in vitro* and *in vivo* effect (the latter after injection into the host), there was no effect in this study. This might be due to the solubility of this compound, resulting in non-ingestion by *Artemia,* preventing exposure of this compound to cells responsible for immunostimulation. Sritunyalucksana *et al.* (1999) assesed the effect of Laminarin *in vitro* by measuring PO, agglutinin and antibacterial activities in black tiger shrimp. Interestingly, their results showed a reduction in PO and antibacterial activities following Laminarin treatment. Furthermore, Muñoz *et al.* (2006) reported no significant effect of using Laminarin on haemolyph PO activity in three clam species. In freshwater crayfish *Astacus astacus,* injections with laminarin resulted in increased levels of prophenoloxidase mRNA in the haemocytes, whereas the levels of several other transcripts such as

actin or the blood cell adhesion protein peroxinectin remained unchanged (Cerenius *et al.,* 2003). In standard infection experiments conducted with the fungal parasite, *Aphanomyces astac*i, the accumulated mortality reached 50% within 4 days in the infected control crayfish, whereas the same mortality was reached 9 days in the immune-stimulated animals. In summary, literature data and the results reported here suggest that the immunostimulatory effect of Laminarin is dependent on the experimental set-up and further experiments are needed to clarify under which conditions Laminarin can be beneficial to the host.

In the present study the application of two commercial β-glucans namely Biorigin and Immunowall had no favorable effect on *Artemia* nauplii challenged with pathogenic VC (Table 6.3 and 6.4; lines; 12 and 16 vs 18). Although the level of β-glucan in Biorigin is even higher than in MacroGard (see Table 6.2), for unknown reasons it could not contribute to pathogen resistance.

Immunomodulatory effects of chitin (Chitin is a β-1,4-linked polymer of N-acetyl-D-glucosamine and a common constituent of insect, exoskeleton, crustacean shells and fungal cell walls (Esteban *et al.,* 2000) and chitosan have been reported by many workers (Sakai *et al.,* 1992; Anderson *et al.,* 1995). The present study showed no significant effect of chitin in the *Artemia* challenge test (Table 6.3 and 6.4; lines; 6 vs 18). Furthermore, addition of chitin to the β-glucan (Sigma), could not improve the level of pathogen resistance obtained by β-glucan (Sigma) itself. White shrimp, *Litopenaeus vannamei* that have been injected with chitin at 6 µg/g, showed increased phagocytic activity and resistance against *Vibrio alginolyticus* infection (Wang and Chen, 2005)**.** It is known that many external (environmental) and internal factors may influence the effects of a particular immunostimulant (Anderson, 1996). One such factor is the way in which the immunostimulant is administered. In *Penaeus monodon* shrimp, only intramuscular injection of a WSSV (white spot syndrome virus) envelope protein (VP19)- in contrary to oral administration- could improve survival after WSSV challenge (Witteveldt, 2005). Therefore, it is suggested that ingestion of chitin is not a proper way to induce the immune system in gnotobiotic *Artemia*.

In the present study, VC never had a significant negative impact on individual growth. However in combination with the survival values, total biomass production was always significantly lower compared to the respective controls, indicating that VC still had a adverse effect on *Artemia*, even in the presence of a protective agent like glucan (Sigma) and zymosan (Table 6.3 and 6.4, treatments: 1 vs 2, and 7 vs 8). We can only speculate on the mechanism involved. VC could possibly influence food conversion rate (FCR) for instance through a reduced resorption of nutrients or through an increased transit in the gastro-intestinal tract. Alternatively VC could affect the food uptake rate (FUR) resulting in poorer performance. A more targeted study would be needed to verify in which way total biomass production is affected even in the absence of a significant reduced survival.

The values in Table 6.2 indicate that the sugar concentration is different in the various compounds applied in this study. Also, the level of β-glucan within the sugar fraction varies between these products. Therefore, the total amount of β-glucans (dry weight) offered to *Artemia* was calculated (see Table 6.1). In fact, different amounts of β-glucans from these compounds were supplied to *Artemia*. Therefore, one could expect that the differences observed in this study could actually be due to different amounts of β-glucans (from different products) being supplied to *Artemia*. For instance, if Immunowall could not protect *Artemia* in challenge test, it could be argued that this is due to the low β-glucan concentration in this product (offered to *Artemia*) in comparison to Zymosan or β-glucans (Sigma). However, when higher amounts of the products (up to 3 fold) were provided to *Artemia*, the results did not change. In addition, compared with Zymosan or β-glucan (Sigma), the higher amount of β-glucan existing in Biorigin could not provide protection in challenged nauplii. Also, no correlation could be found between chemical composition of the tested compounds and their protective effect in the challenged group. Therefore, it can be postulated that the immunostimulation is not only dependent on the amount of β-glucan offered, but rather the quality (molecular weight, three dimensional structure, type and frequency of branches (Chang *et al.,* 2003) of the products must be important. Interestingly, it was shown that the adsorption affinity

of β-glucans to mycotoxins (*e.g.* Zearalenone (ZEN) produced by numerous *Fusarium* species) is highly dependent on three-dimentional structure of these products (Yiannikouris *et al.,* 2004).

5. Conclusion

This is the first time that these different commercial glucans and chitin particles have been tested simultaneously in a standardized *Artemia* challenge test. Because of the gnotobiotic conditions, this system can be used as a unique tool for testing anti-infectious properties of a specific compound with limited nutritional interference. Therefore, this challenge test, complemented with other tools such as measurement of immune parameters and gene expression analysis can provide further documentation on the exact impact of putative immunostimulants on immunity and disease resistance in *Artemia*, probably offering further insight into the innate immune response in crustacean.

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CHAPTER VII

GENERAL DISCUSSION

AND

FUTURE RESEARCH

General discussion and future research

In recent years, microbial disease has emerged as one of the most important factors hampering the development of aquaculture, being responsible for immense mortalities occurring especially in the early life stages of aquatic organisms. Since a disease outbreak can be difficult to control, farm management focuses on disease prevention. Prevention techniques mainly include control of the microbiota, maintenance of a healthy microbial environments in larviculture, careful selection of broodstock, vaccinations, reduction of stress by adjusting stocking densities, veterinary certification, antibiotic treatments, and fallowing of farms.

The current debate on methods to control disease in aquaculture concentrates on the potential negative effects of some of these treatments, such as the proliferation of antibiotic-resistant bacteria or unintended effects (*e.g.* environmental and human health consequences (Witte, 2000)). To avoid these problems, more environmental-friendly prophylactic and preventive solutions are required (*e.g.* nutritional improvements, probiotics and immunostimulants). However, the quality, effectiveness and reproducibility of such new techniques must be evaluated under standardized and controlled conditions (Smith *et al.,* 2003). Marques *et al*. (2004) have recently developed and validated the usefulness of an *Artemia* gnotobiotic test system, allowing studying the effect of food composition on *Artermia* performance (survival and growth) either in the presence or absence of a pathogen (such as *Vibrio*).

I. Gnotobiotic *Artemia* test system

Several particular characteristics of *Artemia* make this animal very suitable to be used as a test organism, such as small size, short generation time, cyst production and continuous non-selective particle filter-feeder. Marques *et al.* (2004) optimized a gnotobiotic culture of *Artemia*, using a simple and highly reproducible procedure to obtain axenic animals. In a laminar flow hood, cysts were decapsulated using strong oxidizing agents. After the decapsulation procedure, germ-free cysts were obtained and nauplii were allowed to hatch in sterile conditions and to develop until the 2nd

instar stage. At that stage they start ingesting feed. These nauplii were used for setting up the experiments. In order to check for contaminations, a combination of plating on marine agar (MA) and live counting (using tetrazolium salt MTT live stain) was used, following the procedures described in Chapters III. An important consideration, when using gnotobiotic *Artemia* test (GART), is that all media used to culture either *Artemia* or live feeds need to be filtered (0.22µm) before autoclavation in order to remove any microbial biomass (dead or alive), as dead bacteria even when present in small amounts, can considerably alter the nauplii performance and interfere in reproducibility of the experiments (Marques *et al.*, 2005).

Provided they can be cultured axenically, several feed can be used to culture gnotobiotic *Artemia* such as microalgae, live or dead bacterium and baker's yeast *Saccharomyces cerevisiae* (Marques *et al.,* 2005).

In the last decades yeast has been subject to intensive genetic research (*e.g.* its entire genome has been sequenced and genetic manipulation is possible allowing for the construction of gene deletion mutants, as well as strains overexpressing certain genes). Yeast strains harbouring null mutations in genes involved in cell wall synthesis are nowadays commercially available.

Scope of the research

The aim of the present study was to verify the protective nature of baker's yeast strains and extracts in a gnotobiotic *Artemia* test system and to identify the cell-wall components that are conducive to the protective effect against the pathogen in *Artemia* (findings obtained in chapter III, IV, V and VI). Therefore, the effect of isogenic yeast deletion mutants (7 strains) (together with wild type yeast (WT)) and some commercial yeast extracts was evaluated in GART.

II. Host-microbe interaction

Using GART, the outcome of the host-microbial (*Artemia* versus *Vibrio*) interaction was tested under different feeding regimes, such as: (i) different axenic yeast strains; (ii) live and dead bacteria; and (iii) different putative immunostimulants.

2.1. Yeast mutants

In previous studies, Coutteau *et al.* (1990) considered live baker's yeast to have a low feeding value for *Artemia*, likely due to the absence of digestive enzymes in their gut necessary to digest the mannoprotein outer layer of the yeast cell wall. However, the nutritional value of yeast was significantly improved when feeding yeast cells i) harbouring null mutants for enzymes involved early in the biochemical pathway for cell wall mannoproteins synthesis (*e.g.* MNN9 mutation), ii) with a reduced possibility to phosphorylate mannoproteins (*e.g.* MNN6 mutation), iii) with reduced chitin (*e.g.* CHS3 mutation) or reduced glucan contents (*e.g.* FKS1, KNR4, KRE6 and GAS1 mutation). In addition, the yeast growth phase and yeast growth medium were found to be important parameters to determine the quality of yeast cells as feed for *Artemia*. A strong positive correlation was found between *Artemia* performance and the yeast cell wall chitin and glucan contents, while the mannoprotein content was negatively correlated with *Artemia* performance (see Chapter III). Live mnn9 yeast cells grown on yeast nitrogen base (YNB) medium and harvested in the exponential growth phase proved to be an excellent yeast feed for *Artemia* (Marques *et al.,* 2004). In conclusion, these results suggest that any mutation affecting the yeast cell-wall make-up is sufficient to improve the digestibility for *Artemia.* The yeast cell wall composition, especially higher amounts of cell wall bound chitin and glucans in combination with lower amounts of mannoproteins, play an important role in the enhancement of the yeast digestibility by *Artemia*, thus supporting the hypothesis formulated by Coutteau *et al*. (1990), that external mannoproteins layer might be hard to digest for *Artemia*.

In a second set of experiments, the protective nature of yeast mutants was investigated using GART by exposing *Artemia* to a virulent pathogen (*V. campbellii).* In previous studies, using only yeast cells as food, the protective effect against this pathogen was found to be especially high when

Artemia was fed a good quality food (*e.g.* mnn9 yeast), suggesting that the observed protection was either due to a nutritional effect or an immunostimulatory effect (or a combination). In the present study, in order to try to differentiate between the nutritional effects and the immunostimulatory nature, only a low amount of yeast cells (from 8 strains) was fed to *Artemia* (yeast constituted only 10% of the AFDW offered). Even under these circumstances mnn9 yeast cells still supported an increased individual growth as well as an induced total protection against *Vibrio*. Therefore, it was suggested that, mnn9 yeast has both strong nutritional and protective characteristics (the latter possibly through immunostimulation) (see chapter III). Interestingly, in contrast to mnn9 yeast, the temporary protection obtained by feeding the chs3 strain (at 10% of the total AFDW offered) is not coupled with a better individual growth performance suggesting absence of nutritional interference in dealing with the pathogen. Chitin plays a key role in yeast cell growth and division and is attached covalently to β-1,3-glucans, β-1,6-glucans and mannoproteins (Cabib *et al.,* 2001). Therefore, the better results obtained with nauplii fed chitin defective yeast could be due to an enhanced digestibility of chs3-cells by *Artemia*, caused by reduced linkage between the three cell wall components, eventually increasing the availability of glucan. It is however also possible that the glucan structure is different in the chs3 strain. Anyhow, these results seem to suggest that chitin as such is not involved in the protection against VC (see also Chapter VI).

Since the challenge results obtained with the chs3 strain seem to suggest a pure immunostimulatory effect (no interfering nutritional effect), future research on gene expression in *Artemia* under these circumstances could help in identifying differentially expressed genes, elucidating the immunostimulatory activity of the chs3 strain.

The hypothesis formulated by Coutteau *et al.* (1990) concerning low digestibility of yeast cells by *Artemia* was evaluated in chapter IV, where various yeast strains (8 strains) were treated with 2-mercapto-ethanol (2ME) and then used to asses their nutritional and anti-infectious characteristics for gnotobiotically-grown *Artemia.* In most cases, chemically treated yeast cells were better feed for *Artemia* than the untreated cells. In addition, 2ME treatment of some yeast strains (*e.g.* gas1, kre6 and chs3) significantly improved *Artemia* protection against VC. It is claimed that 2ME cleave the

disulfide linkages in the cell wall mannoproteins, making them more permeable and susceptible to enzymatic degradation in the digestive system of *Artemia* (Coutteau *et al.*, 1990). Possibly, in those treated yeast strains, with improved *Artemia* performance in comparison to untreated cells, the gene mutations affecting the yeast cell wall composition and scaffolding (by reducing the amount of covalent links between the major components of yeast cell wall namely mannoproteins, β-glucans and chitin), predisposed the yeast cell wall to further changes caused by 2ME treatment, resulting in a higher bioavalibility of β-glucan. Taken together, the present study and the results of Coutteau *et al.* (1990) suggest that, apart from the cell wall composition, the type and/or density of covalent links between the three major yeast cell wall components, as well as any weakening of yeast cell wall scaffolding may be responsible for a positive effect on *Artemia* performance in the presence or absence of challenge. Future studies could be performed to generate viable double yeast deletion mutants. In this way it could be possible to further manipulate the composition of the yeast cell wall, identifying which cell wall components are responsible for the immunostimulatory effect in *Artemia.*. Another possibility could be to extract the β-glucan from the yeast strains (single or double mutants) and to test them in GART system. Interesting findings were also described in chapter V, where either protective yeast cells (mnn9 yeast) or a putative immunostimulant (*e.g.* glucan particles) were supplied to *Artemia* at different time points with respect to the VC challenge. That study revealed that these compounds could only provide protection against the pathogen when they were supplied to *Artemia* well in advance of a challenge (8 to 48h depending the source). In addition, the putative immunostimulants did not have a clear curative effect. The fact that mnn9 yeast could trigger the protective effect faster than pure glucan could be due to two phenomena. Either, mnn9 cells provided extra nutritional compounds not present in pure glucan which is supported by the feeding trials or the induction of better individual growth obtained by feeding this yeast strain, or it is also possible that the induction of the protective effect by the complete yeast cells may be better because the immune system has evolved over time and learned to recognize glucan embedded in a (yeast) cell wall matrix better then chemically pure glucan. In addition the chemical nature of the glucans (*e.g.* the ratio of β-1,3 and β-1,6-glucan, the molecular weight, the

dimensional structure, type and frequency of branches) can also have a major effect (Ai *et al.,* 2007). Hence this calls for a more detailed characterization of the i) yeast cell wall extracts (such as commercial β-glucans) as well ii) the cell wall structure of the used yeast strains. Some advocates of glucan or other immunostimulants argue that treatment with these substances cause a fast immune reaction (*i.e.* release of immune molecules such as opsonin, binding molecules and other defense proteins into the circulation) against opportunistic or pathogenic invasion. However, there is little unequivocal evidence that the so-called 'immunostimulants' act in this way. In crayfish, fungal infection can be mimicked with injection of a β1,3-glucan (*e.g.* laminarin) which induces a rapid drop in haemocyte number followed by a recovery after 24–48 hours (Söderhäll *et al.,* 2003). The loss of circulating haemocytes after β-1,3-glucan injection is probably due to cell aggregation inside the animal, indicating an important role of the haemocytes in defense. It was shown in *Fusarium*-infected shrimp, that the rate of haemocytes proliferation significantly increased. Therefore, fungal infection or β-1,3-glucan administration not only affects hemocytic behaviour, as earlier described for freshwater crayfish (Persson, 1987; Thomqvist, 1993), but also haemocyte proliferation and perhaps haemocyte synthesis or metabolism are affected. The time elapse between treatment and response appears to vary with the species and the product used. With *P. japonicus* a three-fold increase in the number of circulating haemocytes were observed 5 days after injection of LPS and a six-fold increase after injection of the fungus, *Fusarium.* Therefore, under the described experimental circumstances glucan and/or mnn9 yeast (after uptake and probably ingestion) are likely triggering the proliferation or the maturation of the haemocytes, explaining the time lag between the addition of the putative immunostimulants and the appearance of the protective effect. This hypothesis needs careful experimental verification. If proliferation of heamocytes could be demonstrated, the used yeast cells or glucans would become candidate immunostimulants for *Artemia* (and probably for other crustaceans). It should be noticed that the protective effect obtained by these products in newborn *Artemia* nauplii may not necessarily be as strong in more advanced developmental stages such as juvenile or adult *Artemia***,** especially in open culture, where *Artemia* will have been exposed to a variety of microbial cell wall material. Unfortunately, little is

known about the ontogeny of the immune system in invertebrates and that of *Artemia* in special. Nevertheless, a limited number of reference have shown that immune capabilities vary depending on different developmental stages of an organism.

In the present study short-time exposure of *Artemia* to mnn9 strain (priming) did not provide a long-time protection against the pathogen. Previous priming studies with the mealworm beetle, *Tenebrio molitor*, revealed a long-term protection (50 days) against the pathogenic fungus *Metarhizium anisopliae* after priming the innate immune system with lipopolysaccharides (LPS) (Moret and Siva-Jothy, 2003). Although beetles are phylogenetically distant from *Artemia* and the experimental conditions in the present study are different, this is the only literature available which could be linked to the current study. On the whole, the priming of the innate immune system of invertebrates could depend on several factors such as the specificity and quantity of a compound used as well as the animal tested.

2.2. Enhancement of disease resistance

Immunostimulants are claimed to demonstrate positive impact on immunity and disease resistance in aquaculture. However, there are still many doubts on the efficacy of these compounds, especially in invertebrates (Smith *et al.*, 2003). According to the latter authors, there is an urgent need for proper evaluation of the efficacy of putative immunostimulants in more standardised trials, under a range of rearing conditions and with a diversity of pathogens. The use of gnotobiotic *Artemia* can be an excellent tool to evaluate the efficacy of immunostimulants in standardized conditions.

Therefore, in Chapter VI, the protective potential of a series of commercial β-glucans and chitin particles was verified in gnotobiotic *Artemia* fed with poor-quality feeds (dead bacterial biomass). This feed was not able to provide protection against the pathogen. No growth enhancement (nutritional effect) was observed among the products. The results showed that, only β-glucan (Sigma) and Zymosan could completely protect the *Artemia* against the pathogen while MacroGard provided some level of protection. *Artemia* not supplemented with those glucan particles displayed high mortality. No protective effect was observed with chitin particles. Although the exact reason

remains not clear, it could be that ingestion of chitin is not a proper way to induce the immune system in gnotobiotic *Artemia,* as injection of chitin in white shrimp, *Litopenaeus vannamei* increased resistance against *Vibrio alginolyticus* infection. It has been reported that, different administration protocols (*e.g.* immersion, injection, oral) have produced different results, even with the same substance (Witteveldt, 2005; Azad *et al.,* 2005). In the present study, a poor correlation was found between survival values of the challenged *Artemia* and the product compositions (such as chitin, mannose and β-glucan content) indicating that the quality of β-glucans (*e.g.* the ratio of β-1,3 and β-1,6-glucan, the molecular weight, the dimensional structure, type and frequency of branches), eventually in combination with other unidentified compounds is more important than the amount of product offered.

In conclusion, the immunomodulatory effects of glucans are not unequivocal and have been shown to be different depending on the product source, animal species, development stage of the target organism, dose and type of glucan, route and time schedule of administration (Guselle *et al.,* 2007) and the association with other immunostimulants. Moreover, the impact of immunostimulants on the developing immune system of *Artemia* is not clear. In fish larvi, some researchers maintain that the effect is minimal and immunostimulants can be fed to larval fish as soon as the animal can be weaned to an artificial diet (Bricknell and Dalmo, 2005). However, it is also believed that administering potentially powerful immunomodulating compounds to an animal that is still to experience major developmental changes in the immune system results in the induction of tolerance against immunostmulants (Bricknell and Dalmo, 2005). Therefore, further studies should be performed in standardized conditions (*e.g.* using gnotobiotic *Artemia*) to evaluate the potential impacts of immunostimulants on the developing immune system of target organisms.

Further research on the relationship between gene expression and survival of challenged nauplii in gnotobiotically-grown *Artemia* could elucidate in detail the mechanism or action of yeast cells and/or specific compounds on the innate immune system. Having characterized molecular markers for immunostimulation in *Artemia*, making use to the various treatments that seem to protect

Artemia against *V. campbellii*, it will be necessary to confirm the usefulness of these markers in other species. Proof for this would make the GART system a good model system.

III. Conclusion

The gnotobiotic *Artemia* culture system is a useful tool to evaluate new treatments for disease prevention in aquaculture especially in the early larval stages. In combination with immunological markers it could allow for a very detailed study on the mechanisms involved in host-microbial interactions, contributing to the development of effective solutions for disease control in aquaculture.

The gnotobiotic *Artemia* test system (GART), combined with yeast strains presenting different cell wall composition, presents a unique opportunity (because of no interference of other microbial compounds) to investigate how the yeast cell wall composition influences both macro parameters (*e.g.* growth and survival), as well as gene expression in the host. The identification of genes of importance to the immune function in *Artemia*, followed by the verification of the importance in disease prevention of their heterologous counterparts in other crustaceans, could make the GART system a valid model for developmental immunological studies in invertebrates of economical importance in aquaculture.

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SUMMARY/SAMENVATING

SUMMARY

World aquaculture has grown significantly during the past half-century due to a global increase in demand of fish and shellfish products and to a stagnation in the world fisheries capture. In spite of the technological improvements, intensive culture of aquatic organisms often leads to unpredictable high mortalities, especially in the early life stages, mostly caused by pathogenic or opportunistic bacteria. Until recently, several chemotherapies, such as antibiotics, were considered for controlling these microorganisms in aquaculture. However, this practice is now questioned due to the appearance of several environmental and human disorders (see Chapter I). Nowadays, several new alternative, environmental-friendly prophylactic and preventive strategies are being developed to control diseases and maintain a healthy microbial environment in larvae culture, such as improvements in larval nutrition and the use of probiotics, vaccines and immunostimulants (see Chapter I and II). Nevertheless, the quality, effectiveness and reproducibility of such new techniques must be evaluated under standardized and controlled conditions taking into account ecological, environmental, zootechnical and nutritional aspects. Gnotobiotic aquatic systems, *i.e.*, animals are reared in a germ-free (axenic) state or harbouring a known pre-specified flora of microorganisms, can be excellent tools to investigate several issues (see Chapter II). Gnotobiotically-raised brine shrimp *Artemia* has been developed as an aquatic test system to evaluate new treatments of disease control and to study the mechanisms involved in such control. This test system allows studying the effect of food composition as well as host-microbe interactions. Baker's yeast *Saccharomyces cerevisiae,* which has been found to be a good immune enhancer in some aquatic organisms, is an excellent source of β-glucans and chitin. These compounds, which are well-known as immunostimulants in aquatic organisms, are mainly present in the yeast cell wall as major compounds together with mannoproteins. The general objective of the present thesis was to study the anti-infectious characteristics of isogenic yeast mutants in a

gnotobiotically-grown *Artemia* test system in order to identify the critical cell-wall components involved in the protection against *Vibrio campbellii*. Various yeast cell wall mutants provide a range of cell wall compositions (β-glucans, mannoproteins and chitin). In a first set of experiments (Chapter III), a collection of yeast mutant strains was used to evaluate their nutritional and anti-infectious nature for gnotobiotically-grown *Artemia.* Yeast cell-wall mutants were always better feed for *Artemia* than the isogenic wild type mainly because they supported a higher survival but not necessarily a stronger individual growth. These results suggest that any mutation affecting the yeast cell-wall make-up is sufficient to improve their digestibility for *Artemia.* The second set of experiments investigated the use of a small amount of yeast cells (10% of total AFDW usually provided to nauplii) in gnotobiotic *Artemia* to overcome pathogenicity of *Vibrio campbellii* (VC). Among all yeast cell strains used in this study, only mnn9 yeast (harbouring less cell wall-bond mannoproteins and more glucan and chitin) seems to completely protect *Artemia* against the pathogen. Incomplete protection against the pathogen was obtained by the gas1 and chs3 mutants, which are lacking the gene for respectively a particular cell wall protein and chitin synthesis. However, only with the chs3 strain, in contrast to the gas1 or mnn9 strains, the temporary protection against VC is not concomitant with a better growth performance suggesting non-interference of general nutritional effects.

In Chapter IV, the phenomenon of low yeast digestibility reported by Coutteau was studied. According to these authors, mannoproteins present in the yeast cell wall might be the main barrier for the yeast digestibility by *Artemia*. Therefore, in Chapter IV, the yeast cells (the same strains used in Chapter III) were chemically treated with 2-mercapto-ethanol (2ME) (which is able to reduce and cleave the disulfide links between the cell wall mannoproteins) and then used to evaluate their nutritional and anti-infectious nature for gnotobiotically-grown *Artemia.* In most cases, chemically treated yeast cells were better feed for *Artemia* than the untreated cells. The better performance of *Artemia* fed 2ME-treated cells was due either an increased survival (with WT, knr4 and chs3) or a bigger individual length (fks1 and kre6) or due both to an improved survival and a bigger individual length (mnn6). The 2ME yeast treatment significantly improved *Artemia*

protection against VC when applied to gas1, kre6 and chs3 strains. It is postulated that simple chemical treatment in these strains could possibly increase the bio-availability of β-glucans to *Artemia* stimulating the immune system against the pathogenic VC. So, it can be stated that certain yeast mutants (treated or untreated with 2ME) could be considered as promising immunostimulants in aquaculture.

It has been previously shown that small quantities of the mnn9 yeast cells and/or glucan particles could protect *Artemia* nauplii against the pathogenic bacterium *Vibrio campbellii* in the gnotobiotic *Artemia* challenge test. Apparently, the higher amount and/or availability of β-glucans and/or chitin present in mnn9 yeast strain might play an essential role in such protection. The present study (see Chapter V) reveals that these compounds could only provide protection against the pathogen when they were supplied to *Artemia* well in advance to the challenge (8 to 48h depending on the source, mnn9 and glucan respectively). Also the putative immunostimulanthardly have a curative action. Although some reports argue that β-glucan or other immunostimulants cause a fast immune reaction (*i.e.* release of immune molecules such as opsonin, binding molecules and other defense proteins into the circulation) (which was not observed in this study) against pathogens, there is little unequivocal evidence to support this hypothesis. However, it has been reported that β-glucan administration not only affects hemocytic behaviour but also haemocyte proliferation and perhaps haemocyte synthesis or metabolism are affected. The time needed for the response to appear varies with species, developmental stage of the animal and the product used. Therefore, it can be postulated that the β-glucans in mnn9 cells are probably more appropriate than pure β-glucan (*e.g.* the ratio of β-1,3 and β-1,6-glucan, the molecular weight, the dimensional structure, type and frequency of branches) which enables the mnn9 cells to trigger the immune system (probably through haemocyte proliferation) in *Artemia* in a shorter period of time compared with pure β-glucan.

In the present study (Chapter V) short-time exposure of *Artemia* to mnn9 strain (priming) did not provide protection against the pathogen longer than 2 days. Although the exact reason is not clear

yet, it has been reported that the priming of the innate immune system of invertebrates could depend on several factors such as the specificity and quantity of a compound used as well as the animal tested.

Immunostimulants are claimed to demonstrate positive impact on immunity and disease resistance in aquaculture. However, there are still many doubts on the efficacy of these compounds, especially in invertebrates. Therefore, in a last set of experiments (Chapter VI) the anti-infectious potential of some putative immunostimulants was verified in a gnotobiotic *Artemia* challenge test system. Therefore, six commercial β-glucans (all obtained from baker's yeast *Saccharomyces cerevisiae* except for Laminarin) and chitin particles were verified in gnotobiotic *Artemia* fed with poor-quality and non-protective feeds and challenged with pathogenic VC. The results showed that, only β-glucan (Sigma) and Zymosan could totally protect the *Artemia* against the pathogen while MacroGard provided some level of protection. *Artemia* not supplemented with those glucan particles displayed high mortality.

In conclusion, the gnotobiotic *Artemia* culture system (GART) is an excellent tool to evaluate new treatments and to study the mechanisms involved in host-microbial interactions (if combined with immune markers, that still need to be developed for *Artemia*). It is hoped that GART can in this way contribute to the development of effective solutions for disease control in aquaculture.

SAMENVATTING

De aquacultuur productie is wereldwijd de voorbij decennia beduidend toegenomen door de globale toename van de vraag naar vis en schaaldieren en door de stagnatie van het aanbod uit de visserij. Ondanks de technologische vooruitgang, lijdt de intensieve kweek van aquatische organismen dikwijls aan onvoorspelbare en hoge sterfte, voornamelijk gedurende de eerste levensstadia, en dit hoofdzakelijk door pathogene of opportunistische bacteriën. Tot voor kort werden chemische therapieën, zoals antibiotica, aangewend om de micro-organismen onder controle te houden. Deze praktijken zijn nu grotendeels verbannen wegens de implicaties voor het milieu en de menselijke gezondheid (Hoofdstuk I). Momenteel worden er verscheidene alternatieve en milieuvriendelijke profylactische en preventieve strategieën ontwikkeld voor ziektebeheer en controle van de microbiologische omgeving in de larventeelt, met name de verbetering van het larvale voedsel, het gebruik van probiotica, vaccinatie en immunostimulantia (Hoofdstuk I en II). De kwaliteit, reproduceerbaarheid en efficiëntie van deze nieuwe technieken moet wel geëvalueerd worden onder gecontroleerde standaard omstandigheden rekening houdend met ecologische, zoötechnische en nutritionele aspecten. Gnotobiotische aquatische systemen, waarbij dieren gekweekt worden in een omgeving waarbij geen of enkele specifieke en gekende micro-organismen aanwezig zijn, zijn een uitstekend hulpmiddel om dit te bestuderen (Hoofdstuk II). Een gnotobiotisch kweektest voor *Artemia* werd ontwikkeld om nieuwe behandelingen tegen ziektekiemen te bestuderen alsook de mechanismen die bij die behandeling betrokken zijn. Dit testsysteem laat toe om de effecten te bestuderen van bepaalde nutritionele bestanddelen en gastheer-bacterie interactie.

Bakkersgist, *Saccharomyces cerevisiae,* blijkt een goede immunostimulant te zijn voor bepaalde aquatische organismen en is een uitstekende bron van β-glucanen en chitine. Deze componenten komen vooral voor in the gist celwand samen met mannoproteïnen. De algemene doelstelling van deze studie is het onderzoek naar de beschermende werking van isogene gistmutanten voor *Artemia* tegen pathogene bacteriën en de identificatie van celwand componenten verantwoordelijk voor de

beschermende werking. Dankzij de uiteenlopende celwand samenstelling van de gebruikte gist mutanten (verhouding van β-glucanen, mannoproteïnen en chitine), kon in de eerste reeks experimenten (Hoofdstuk I) de nutritionele en beschermende eigenschappen ten opzichte van *Artemia* worden onderzocht. De gist mutanten bleken steeds een beter voedsel te zijn voor *Artemia* dan de isogene niet-gemuteerde gist, voornamelijk door de hogere overleving en in mindere mate door een betere groei. Dit resultaat suggereert dat ongeacht welke mutatie de celwand structuur van gist beïnvloedt, dit volstaat om de verteerbaarheid door *Artemia* te bevorderen.

Een tweede reeks experimenten onderzocht het gebruik van minimale toevoegingen van gistcellen (10% van het totale asvrije drooggewicht dat werd toegediend als voedsel) voor het verkrijgen van bescherming tegen pathogene Vibrio campbellii (VC). Van alle onderzochte gistmutanten in deze studie alleen *Artemia* gevoed met mnn9 (stam met minste celwand gebonden mannoproteïnen en meer glucanen en chitine) een volledige bescherming tegen de pathogeen bieden. Gedeeltelijke bescherming tegen de pathogeen werd bereikt met de gas1 en chs3 mutant, de welke het gen ontbreken om resp. een specifieke celwand proteïne en chitine te synthetiseren. In tegenstelling met de gas1 en mnn9 gist stam ging de bescherming tegen de pathogene VC door de chs3 stam niet gepaard met een betere groei hetgeen suggereert dat er geen nutritioneel effect in het spel was.

In Hoofdstuk IV werd de beperkte verteerbaarheid van gist door *Artemia* nader bestudeerd. Volgens Coutteau et al. (1990), vormen mannoproteïnen in de celwand van gist the belangrijkste belemmering voor de vertering door *Artemia*. In Hoofdstuk IV werden de gistmutanten chemisch behandeld met 2-mercapto-ethanol (ME) en dan gevoederd aan *Artemia* om de nutritionele en immonostimulerende effect te evalueren. In de meeste gevallen was de behandelde gist een beter voedsel voor *Artemia*. De betere prestatie van *Artemia* met de behandelde gisten was te danken, hetzij door een betere overleving (met WT, knr4 en chs3), hetzij door een betere groei (fks1 en kre6), hetzij door zowel betere groei als overleving (mnn6). De ME behandeling verbeterde significant de bescherming tegen de pathogeen bij gas1, kre6 en chs3 stammen. Vermoedelijk verbeterde de chemische behandeling de beschikbaarheid van β-glucanen voor *Artemia* en stimuleerde zodoende het immuunsysteem tegen de pathogene VC.

Eerder werd aangetoond dat een geringe dosis mnn9 gisten/of glucan partikels *Artemia* bescherming bood tegen VC in een gnotobiotische kweektest. Blijkbaar kan een hogere dosis en/of hogere beschikbaarheid van β-glucanen en/of chitine aanwezig in mnn9 een doorslaggevende rol spelen in de bescherming. Deze studie (Hoofdstuk V) toonde aan dat deze componenten bescherming boden tegen VC enkel wanneer ze werden toegediend ruim voor de besmetting (8 tot 48h). Verder had de vermeende immunostimulant geen curatief effect. Hoewel enkele studies beargumenteren dat β-glucanen of andere immunostimulantia een snelle immuunreactie veroorzaken tegen pathogenen is er weinig eensluidend bewijs voor deze hypothese. Er wordt vermeld dat β-glucaan niet alleen het haemocytisch effect beïnvloed maar ook de proliferatie en mogelijks ook de synthese of metabolisme van haemocyten, terwijl de responstijd varieert met het species, het ontwikkelingsstadium en het gebruikte product. Daarom kan er gesteld worden dat β-glucanen in de mnn9 gistmutant meer geschikt zijn dan pure β-glucanen en dat mnn6 sneller het immuunsysteem induceert dan pure glucanen.

In deze studie (Hoofdstuk V) werd aangetoond dat een korte blootstelling van *Artemia* aan mnn9 (priming) geen bescherming opleverde tegen het pathogeen langer dan 2 dagen. Hoewel de reden niet gekend is werd aangetoond dat priming van het immuunsysteem van ongewervelden bepaald wordt door verschillende factoren waaronder de specificiteit en hoeveelheid van het product alsook van het geteste organisme.

Immunostimulantia worden geacht een positief effect te sorteren op het immuunsysteem en de weerbaarheid van aquatische organismen. Er heerst echter nog veel onduidelijkheid over deze producten, vooral voor ongewervelden. Daarom werd in een laatste reeks experimenten (Hoofdstuk VI) de anti-ontstekingswerking van enkele vermeende immunostimulantia geverifieerd in een gnotobiotisch Artemia kweeksysteem. Zes commerciële β-glucanen (5 gebaseerd op bakkergist, 1 op Laminarine) en chitine partikels werden getest. De resultaten toonden aan dat enkel β-glucaan (Sigma- en Zymosan totale bescherming tegen VC boden, terwijl MacroGard slechts beperkte bescherming bood. *Artemia* die niet werden gesupplementeerd met glucanen toonden hoge sterfte.

Het gnotobiotische kweeksysteem voor *Artemia* bied een uitstekende mogelijkheid om het mechanisme te bestuderen in de interactie tussen gastheer en bacterie en om alternatieve behandelingen te evalueren hetgeen hopelijk bijdraagt tot de ontwikkeling van afdoende oplossingen voor ziektebestrijding in aquacultuur.

CURRICULUM VITAE

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Siyavash Soltanianwas was born in Tehran, Iran, on June 5th, 1969. In 1994, he obtained a veterinary surgeon diploma (D.V.M) at the Faculty of Veterinary Medicine, Shahid Chamran University of Ahwaz, Iran. Afterward, he was employed as a researcher in Razi Vaccine and Serum Research Institute, Karadj city, Iran.

In 2003, he was awarded a Ph.D scholarship by the Ministry of Science, Research and Technology of Iran. In October 2003, he started his PhD training program on "Protection of gnotobiotic *Artemia* against *Vibrio campbellii* using baker's yeast strains and extracts" at the Laboratory of Aquaculture and Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University, which resulted in the present thesis.

Siyavash Soltanian is author of many articles published/submitted in national or international peer-reviewed journals. He has participated in several international meetings, seminars and workshops concerning aquaculture and fish and shellfish immunology.

MSc thesis supervision

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Siyavash Soltanian, Jean Dhont, Patrick Sorgeloos and Peter Bossier (2007) Influence of different yeast cell-wall mutants on performance and protection against pathogenic bacteria (*Vibrio campbellii*) in gnotobiotically-grown *Artemia.* Fish & Shellfish Immunology **23:** 141-153.

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Passive participation

10th PhD Symposium on Applied Biological Science. Ghent University, Belgium, September 29, 2004.

Workshop " Molecular microbial ecology". LabMet, Ghent University, August 23-26, 2005.

Workshop " Fish Immunology". Wageningen Institute of Animal Science (WIAS), April 15-19, 2007.

Courses which were followed during the PhD study

Course "General Aspects of Aquaculture", organized by Laboratory of Aquaculture and Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University.

Course " Aquaculture Genetics", organized by Laboratory of Aquaculture and Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University.

Course " Health and Disease in Aquaculture", organized by Laboratory of Aquaculture and Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University.

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