ELSEVIER

Journal of Experimental Marine Biology and Ecology
312 (2004) 115–136

Journal of
EXPERIMENTAL
MARINE BIOLOGY
AND ECOLOGY

www.elsevier.com/locate/jembe

Evaluation of different yeast cell wall mutants and microalgae strains as feed for gnotobiotically grown brine shrimp *Artemia franciscana*

Antonio Marques*, Jean Dhont, Patrick Sorgeloos, Peter Bossier

Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Rozier 44, 9000 Ghent, Belgium

Received 14 April 2004; received in revised form 14 June 2004; accepted 19 June 2004

Abstract

The nutritional value of isogenic yeast strains and two microalgal species for gnotobiotically grown *Artemia* was examined. Yeast cell wall mutants were always better feed for *Artemia* than their respective wild type. Yeast cells harbouring null mutants for enzymes involved early in the biochemical pathway for cell wall mannoproteins synthesis performed best as feed for *Artemia*. Yeast cells defective in chitin or β-glucan production were scored in second order. The mnn6 isogenic yeast mutant, harbouring a null mutation for mannoprotein phosphorylation, performed poorly as feed for *Artemia*, although with good growth. These results suggest that any mutation affecting the yeast cell wall scaffolding by reducing the amount of covalent links between the major components of yeast cell wall, namely mannoproteins, β-glucans and chitin, is sufficient to improve the digestibility for *Artemia*. The results with microalgae indicated that within one species, strains can have different nutritional value under gnotobiotic conditions. The growth phase was another parameter influencing feed quality, although here it was not possible to reveal the exact cause. It is anticipated that the standard *Artemia* gnotobiotic growth test is an excellent tool to study the mode of action of bacteria, with a probiotic as well as with a pathogenic character.

Keywords: Artemia; Gnotobiotic culture; *Saccharomyces cerevisiae*; *Dunaliella tertiolecta*

---

* Corresponding author. Tel.: +32 92643754; fax: +32 92644193.
E-mail address: Antonio.Marques@UGent.be (A. Marques).

0022-0981/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jembe.2004.06.008

1. Introduction

The brine shrimp, *Artemia*, is one of the most important live feeds for commercial production of fish and shellfish larvae. This organism is unique in the animal kingdom, because it is extremely euryhaline and can produce cysts (encapsulated embryos), which allow a population to survive when environmental conditions are adverse. These cysts can easily be stored and used as instant live food for aquaculture target species (Sorgeloos et al., 2001).

Artemia is an excellent model organism to study intrinsic feed quality without interference of microbes, as it can be easily cultured in gnotobiotic conditions (Marques et al., 2004). *Artemia* is a non-selective particle filter feeder (Dobbeleir et al., 1980), and can be reared on a wide variety of natural feeds, such as microalgae (e.g. *Dunaliella tertiolecta* and *Tetraselmis suecica*) and baker's yeast (*Saccharomyces cerevisiae*) (Walne, 1967; Sick, 1976; Johnson, 1980; Coutteau et al., 1990; Verdonck et al., 1994; Fábregas et al., 1996, 2001). It has been demonstrated that *Artemia* can feed on bacteria (Intriago and Jones, 1993; Gorospe et al., 1996), but to understand the role of both the feed and microbiota in the development of *Artemia*, it is important to separate the effect of the feed quality from the effect of the accompanying microbiota. *Artemia* performance is usually better with live microalgae than with live baker's yeast, since yeast is less digestible by *Artemia*. According to Marques et al. (2004), yeast digestibility is positively correlated with high levels of chitin and β-glucans and negatively correlated with high levels of mannoproteins present in the yeast cell wall.

The cell wall of *S. cerevisiae* is an essential and complex organelle. Its structure determines cell shape and structural integrity, enables cells to withstand internal turgor pressure, protects cells against environmental stress (De Groot et al., 2001; Lagorce et al., 2003), serves to exclude hydrolytic enzymes (Jungmann et al., 1999) and determines the cell wall's porosity by regulating the leakage of proteins from the periplasmic space and the entrance of macromolecules from the environment (Klis et al., 2002). 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, β-1,6 glucan (8% of cell wall DW) and various mannoproteins (40–50% of cell wall DW), existing mostly in the outer layer (Dallies et al., 1998; Klis et al., 2002; Magnelli et al., 2002; Marques et al., 2004). The mechanical strength of the cell wall is mainly due to the inner layer, while the outer layer is involved in cell–cell recognition events and limits the accessibility of the inner part of the wall and the plasma membrane to foreign enzymes, such as cell wall-degrading enzymes (Klis et al., 2002).

The cell wall is a dynamic structure that can adapt to physiological (e.g. from logarithmic to stationary growth phase), to morphological (conjugation, sporulation or pseudohyphal growth) and to environmental changes (De Groot et al., 2001; Aguilar-Uscanga and François, 2003; Marques et al., 2004).

The present study examines the phenomenon of low yeast digestibility in gnotobiotically grown *Artemia* when fed only live yeast cells, using a series of null mutants harvested in the exponential and stationary growth phase. The mutants carry a null mutation for genes involved in the production of cell wall associated mannoproteins, β-1,3 glucans, β-1,6 glucans and chitin. For comparison purposes, two different strains of *D. tertiolecta* and *T. suecica* were tested as well.

2. Materials and methods

2.1. Yeasts and microalgae axenic cultures

To investigate the problem of low digestibility of live baker's yeast (*S. cerevisiae*) by *Artemia*, 14 different null mutants of yeast (isogenic deletion strains derived from BY4741) and the wild type strain (WT) (genotype described in Table 1) were tested as

Table 1

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

Gene	Genotype	Description of the gene product in the cell wall
OCH1	BY4741; *Mat a; his3Δ1; leu2Δ0*; met15Δ0; ura3Δ0; YGL038c::kanMX4	N-linked glycoprotein maturation and initiating alpha-1,6-mannosyltransferase
MNN9	BY4741; *Mat a; his3Δ1; leu2Δ0*; met15Δ0; ura3Δ0; YPL050c::kanMX4	N-linked glycosylation and alpha-1,6-mannosyltransferase
MNN8	BY4741; *Mat a; his3Δ1; leu2Δ0*; met15Δ0; ura3Δ0; YEL036c::kanMX4	N-linked glycosylation and alpha-1,6-mannosyltransferase
MNN10	BY4741; *Mat a; his3Δ1; leu2Δ0*; met15Δ0; ura3Δ0; YDR245w::kanMX4	mannoprotein biosynthesis, alpha-1,6-mannosyltransferase and mannan metabolism
MNN11	BY4741; *Mat a; his3Δ1; leu2Δ0*; met15Δ0; ura3Δ0; YJL183w::kanMX4	alpha-1,6-mannosyltransferase
MNN2	BY4741; *Mat a; his3Δ1; leu2Δ0*; met15Δ0; ura3Δ0; YBR015c::kanMX4	alpha-1,2-mannosyltransferase
MNN5	BY4741; *Mat a; his3Δ1; leu2Δ0*; met15Δ0; ura3Δ0; YJL186w::kanMX4	alpha-1,2-mannosyltransferase
MNN6	BY4741; *Mat a; his3Δ1; leu2Δ0*; met15Δ0; ura3Δ0; YPL053c::kanMX4	mannosylphosphate transferase
MNN4	BY4741; *Mat a; his3Δ1; leu2Δ0*; met15Δ0; ura3Δ0; YKL201c::kanMX4	mannosylphosphorylation of O-linked oligosaccharide, and N-linked oligosaccharide
MNN1	BY4741; *Mat a; his3Δ1; leu2Δ0*; met15Δ0; ura3Δ0; YER001w::kanMX4	alpha-1,3-mannosyltransferase and O-linked glycosylation
KRE6	BY4741; *Mat a; his3Δ1; leu2Δ0*; met15Δ0; ura3Δ0; YPR159w::kanMX4	beta-1,6 glucan biosynthesis
KNR4	BY4741; *Mat a; his3Δ1; leu2Δ0*; met15Δ0; ura3Δ0; YGR229c::kanMX4	beta-1,3 glucan biosynthesis
FKS1	BY4741; *Mat a; his3Δ1; leu2Δ0*; met15Δ0; ura3Δ0; YLR342w::kanMX4	beta-1,3 glucan biosynthesis
CHS3	BY4741; *Mat a; his3Δ1; leu2Δ0*; met15Δ0; ura3Δ0; YBR023C::kanMX4	chitin biosynthesis
WT	BY4741; *Mat a; his3Δ1; leu2Δ0*; met15Δ0; ura3Δ0	control yeast

feed for *Artemia*. All strains were provided by EUROSCARF, University of Frankfurt, Germany.

Yeast cultures were grown in sterile Erlenmeyers closed with a cotton cap and placed on a shaker in the dark (30 °C; 150 rpm). All strains were cultured in a complete Yeast Extract Peptone Dextrose medium (YEPD), containing yeast extract (Sigma, 1% w/v), peptone bacteriological grade (Sigma, 1% w/v) and D-glucose (Sigma, 2% w/v). This medium was prepared in natural seawater (35 g/l) that was previously filtered (0.22 μm) and sterilized by autoclavation at 120 °C for 20 min.

The growth curve of each strain was established by regularly measuring their absorbance at 600 nm with a spectrophotometer. All strains were harvested by centrifugation (3000 rpm for 10 min), either in the exponential growth phase (after 20 h; "exp-yeast") or in the stationary growth phase (after 3 days; "stat-yeast"). Cells were resuspended twice in sterile Falcon tubes (TRP®, γ-irradiated) with 20 ml of filtered (0.22 μm) and autoclaved seawater (FASW). Manipulations were performed in a laminar flow hood to maintain sterility. Yeast density was determined by measuring twice the cell concentration, using a Bürker haemocytometer.

Axenic cultures of the microalgae *D. tertiolecta* (strains DT CCAP 19/6B and DT CCAP 19/27) and *T. suecica* (strains TS CCAP 66/4 and TS CCAP 66/22A) were obtained from the CCAP department, Dunstaffnage Marine Laboratory, Scotland. According to C.N. Campbell (personal communication), responsible for the microalgae culture collection, there are no records of genotypic or phenotypic differences between the microalgae strains. In addition, for both *D. tertiolecta* strains, a nuclear rDNA internal transcribed spacer region 2 was used to confirm if the sequence of both microalgae strains were identical to already published sequences of other *D. tertiolecta* strains, such as UTEX 999, CCMP 1320, and FHL. Each strain was grown in sterile autoclaved 500 ml bottles (10% inocula) with 0.22 μm filtered aeration at 19 °C, continuous light (10–15 cm from the white fluorescent light—2000 lx), using a standard Walne medium (Walne, 1967) and FASW.

The growth curve of each microalgae strain was obtained (two replicates), by measuring the daily cell density of each culture using a Bürker haemocytometer, complemented with daily measurements of the optical density (O.D.) using a spectrophotometer (λ=600 nm). Microalgae strains were harvested in the exponential or in the stationary growth phase by centrifuging the culture (3000 rpm for 5 min) in the middle of the exponential growth curve or in the beginning of the stationary phase, respectively. Cultures were resuspended in 20 ml FASW in sterile cylindroconical Falcon tubes.

Yeast and microalgae suspensions were stored at 4 °C and used to feed *Artemia* until the end of each experiment.

2.2. Yeast and microalgae ash content and fatty acid composition

To determine the yeast and microalgae ash free dry weight (AFDW), 50 ml of each culture sample were filtered on pre-dried filters (pore size 0.45 μm, two replicates 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 the dry weight and the ash weight. The DW and AFDW of the control (filter only, $n=2$) were subtracted from all samples.

To determine the levels of fatty acids contained in the lipid fraction of microalgae cultures, approximately 2 g wet weight of cells were harvested for fatty acid methyl ester (FAME) extraction, conducted as described by Coutteau and Sorgeloos (1995). For this purpose, all microalgae strains harvested in the exponential growth phase were analysed. Results were compared with stat-grown WT yeast cultured in YEPD and exp-grown mnn9 cultured in YNB obtained by Marques et al. (2004).

2.3. *Artemia axenic culture*

Experiments were performed with *Artemia franciscana* cysts, originating from the Great Salt Lake, Utah, USA (EG® Type, INVE Aquaculture, Belgium). Bacteria-free cysts and nauplii were obtained via decapsulation, using the procedure described by Sorgeloos et al. (1986), i.e. a process whereby the chorion of the cysts is removed by immersion in a hypochlorite solution for a few minutes. During decapsulation 0.22 μm filtered aeration was provided. All manipulations were carried out under a laminar flow hood and all necessary tools were previously autoclaved at 120 °C for 20 min.

Decapsulated cysts were washed carefully with FASW over a 50 μm pore size sterile net and transferred to a sterile 50 ml screw cap Falcon tube containing 30 ml of FASW. The tube was capped and placed on a rotator at 4 cycles per min to prevent clogging and sedimentation of the cysts. Cysts were exposed to constant incandescent light (2000 lx) and 28 °C for 18–20 h.

After 18–20 h, the hatching tube was taken to the laminar flow and 20 hatched nauplii (Instar II) were picked and transferred to new sterile 50ml Falcon tubes containing 30 ml of AFSW, together with the amount of feed scheduled for day 1. After feeding, all Falcon tubes were put back on the rotator. Tubes were transferred to the laminar flow just once per day for feeding. The daily feeding schedule was adapted from Coutteau et al. (1990), who optimised the feeding schedule of *Artemia* using baker's yeast and *D. tertiolecta* as feed (see Table 2). The feeding schedule is intended to provide ad libitum rations, but avoiding excessive overfeeding in order not to affect the water quality in the test tubes. The same feeding schedule of *D. tertiolecta* was used with *T. suecica* to feed nauplii.

Table 2
Feeding schedule of *Artemia* fed on microalgae and baker's yeast added per Falcon tube (FT) (adapted from Coutteau et al., 1990)

Day	Microalgae cells/FT	Baker's yeast cells/FT
1	3×10^6	9×10^6
2	6×10^6	18×10^6
3	6×10^6	18×10^6
4	6×10^6	24×10^6
5	9×10^6	36×10^6

2.4. Methods used to verify axenity

Axenity of feed, decapsulated cysts and *Artemia* culture at the end of each experiment were checked using a combination of plating and live counting. Absence of bacteria was monitored by transferring 100 μl of culture medium to Petri plates with marine agar 2216 (two replicates). Plates were incubated for 5 days at 25 °C. As for the live counting, each sample was stained with tetrazolium salt MTT (-3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, 0.5% w/v) in a sterile recipient (1 part of MTT to 9 parts of sample) and incubated at 30 °C for 30 min. Under a light microscope (1000× magnification), live bacterial detection and counting were performed. The MTT blue stains all viable/living cells remaining in a culture (Sladowski et al., 1993), making the detection of bacterial contaminations in a culture medium easier. Whenever a culture tube was found to be contaminated, the data were not considered for further analysis and the treatment was repeated if necessary.

2.5. Experimental design

In Experiment 1, all live and axenic yeast strains (WT and the 14 null mutants) were harvested in the exponential growth phase and were used to feed axenic *Artemia*. In Experiment 2, stationary-grown live and axenic yeast strains (the same strains used in Experiment 1) were applied as feed for nauplii. Finally, in Experiment 3, two different strains of *D. tertiolecta* and two different strains of *T. suecica* were harvested in the exponential and stationary growth phases and were tested as feeds for *Artemia*. Each feed was tested in four replicates. All experiments were performed twice (A and B), to verify the reproducibility of the results.

2.6. Survival and growth of Artemia

At the end of each experiment (day 6 after hatching), the number of swimming larvae was determined and survival percentage was calculated. The larval stage of live *Artemia* at the end of each experiment varied between nauplii and meta-nauplii, according to the effect of the different treatments. However, for convenience, they were all named nauplii throughout this article. 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). As a criterion that combines effects of survival and growth, the total length (or total biomass production) was determined according to the following equation:

$$\text{Total length (millimeters per Falcon tube or mm/FT)} = \text{number of survivors} \times \text{mean individual length}$$

Values of larval survival (percentage) were arcsin transformed, while values of individual length and total length were logarithmic transformed or square root transformed to satisfy normal distribution and homocedasticity requirements. Differences on survival, individual length and total length of *Artemia* fed with different feeds were investigated with analysis of variances (ANOVA) and multiple comparisons Tukey's range, tested at 0.05 level of probability, using the software Statistica 5.5® (Statsoft).

3. Results

3.1. *Artemia performance fed live yeast cells*

3.1.1. *Effect of yeast genetic background*

Artemia nauplii were fed with 14 different isogenic mutant strains of baker's yeast (*S. cerevisiae*) and compared with nauplii fed WT yeast under gnotobiotic conditions in the absence of bacteria. Results presented in Tables 3 and 4 (results obtained in Experiments 1

Table 3
Experiment 1—average survival (%), individual length (mm) and total length (mm per Falcon tube—FT) of nauplii fed with 13 yeast mutant strains and the wild type strain (WT) harvested in the exponential growth phase

Strains	A			B		
	Survival (%)	Individual length (mm)	Total length (mm/FT)	Survival (%)	Individual length (mm)	Total length (mm/FT)
Outer cell wall changes						
Less mannan						
och1	58±17^{ab}	3.3±0.7^{a}	39.2±12.4^{a}	69±10^{a}	3.6±0.6^{a}	49.6±4.9^{a}
mnn9	46±20^{abc}	2.9±0.4^{ab}	27.1±14.2^{abcd}	44±12^{abcde}	2.8±0.5^{c}	22.6±6.1^{bcd}
mnn8	41±10^{abc}	3.3±0.6^{a}	28.9±5.7^{abc}	30±8^{cdef}	3.5±0.7^{ab}	21.4±5.1^{bcde}
mnn10	61±8^{ab}	2.6±0.5^{bcd}	31.2±3.0^{abc}	65±11^{ab}	2.7±0.4^{c}	34.8±5.2^{abc}
mnn11	23±7^{cd}	2.6±0.4^{bcd}	12.3±3.8^{de}	27±11^{def}	2.5±0.4^{cd}	13.7±7.7^{def}
mnn2	35±8^{bcd}	2.5±0.3^{bcd}	18.1±4.6^{bcde}	14±5^{f}	2.6±0.4^{bcde}	7.1±2.4^{f}
mnn5	39±12^{abcd}	2.8±0.4^{bc}	21.6±5.7^{bcde}	59±20^{abc}	2.8±0.4^{c}	33.4±14.2^{abc}
mnn1	32±17^{bcd}	2.3±0.4^{cd}	14.4±5.7^{cde}	18±13^{ef}	2.1±0.2^{e}	7.1±4.7^{f}
Less phosphomannan						
mnn4	45±6^{abc}	2.5±0.4^{cd}	22.2±3.4^{bcd}	38±6^{bcdef}	2.2±0.4^{cde}	16.5±4.7^{cdef}
mnn6	69±5^{a}	2.5±0.4^{cd}	34.4±1.9^{a}	69±9^{a}	2.8±0.4^{bc}	37.9±5.4^{ab}
Less β-1,6-glucan						
kre6	48±13^{abc}	2.4±0.4^{cd}	25.5±9.4^{abcd}	24±13^{def}	2.4±0.2^{cde}	12.0±7.5^{def}
Inner cell wall changes						
Less β-1,3-glucan						
knr4	39±6^{abcd}	2.1±0.5^{cd}	16.4±2.2^{bcde}	13±9^{f}	2.3±0.5^{cde}	5.5±3.4^{f}
fks1	28±19^{cd}	2.2±0.2^{d}	12.1±8.4^{de}	50±19^{abcd}	2.1±0.4^{de}	20.8±5.9^{bcde}
Less chitin						
chs3	40±7^{abcd}	2.1±0.4^{cd}	16.6±2.9^{bcde}	57±3^{abcd}	2.4±0.4^{cde}	20.1±1.4^{cd}
No changes						
WT	15±4^{d}	2.3±0.2^{cd}	6.6±1.2^{e}	18±6^{ef}	2.3±0.5^{cde}	7.8±2.7^{ef}

Means were put together with the standard deviation (mean±S.D.). Each experiment was repeated twice: A and B. Values in the same column showing the same superscript letters are not significantly different ($p>0.05$). Strains were ordered according with their insufficiency on cell wall components (mannose, phosphomannose and glucans). Within each cell wall component, strains were organized by an increased proximity to the cell wall composition of the wild type strain in terms of mannose and glucans.

Table 4
Experiment 2—average survival (%), individual length (mm) and total length (mm per Falcon tube—FT) of nauplii fed with 13 yeast mutant strains and the wild type strain (WT) harvested in the stationary growth phase

Strains	A			B		
	Survival (%)	Individual length (mm)	Total length (mm/FT)	Survival (%)	Individual length (mm)	Total length (mm/FT)
Outer cell wall changes						
Less mannan						
och1	20 ± 10^{bc}	3.2 ± 0.7^{ab}	13.4 ± 7.2^{bcd}	15 ± 7^{cd}	3.0 ± 0.2^{ab}	9.2 ± 5.8^{cd}
mnn9	49 ± 14^{a}	2.8 ± 0.4^{bc}	29.6 ± 11.3^{ab}	54 ± 8^{a}	3.1 ± 0.6^{ab}	33.9 ± 6.5^{ab}
mnn8	45 ± 4^{ab}	3.5 ± 0.6^{a}	31.6 ± 3.3^{ab}	31 ± 8^{b}	3.3 ± 0.6^{ab}	21.2 ± 6.3^{abc}
mnn10	60 ± 22^{a}	3.2 ± 0.5^{ab}	38.4 ± 14.1^{a}	62 ± 5^{a}	3.1 ± 0.5^{ab}	40.0 ± 5.6^{a}
mnn11	10 ± 9^{cd}	2.9 ± 0.2^{bc}	5.8 ± 5.2^{def}	9 ± 3^{d}	3.4 ± 0.3^{a}	5.9 ± 1.4^{d}
mnn2	4 ± 5^{cd}	1.9 ± 0.2^{cde}	1.4 ± 1.8^{ef}	1 ± 3^{e}	1.5	0.4 ± 0.7^{e}
mnn5	0^{d}	–	0.0^{f}	0^{e}	–	0.0^{e}
mnn1	20 ± 15^{bc}	2.2 ± 0.3^{de}	8.7 ± 6.1^{cde}	31 ± 14^{bc}	2.2 ± 0.3^{c}	13.6 ± 5.9^{bcd}
Less phosphomannan						
mnn4	5 ± 4^{cd}	1.6 ± 0.7^{de}	1.6 ± 1.3^{f}	1 ± 3^{e}	2.2	0.6 ± 1.1^{e}
mnn6	45 ± 12^{ab}	2.3 ± 0.4^{de}	20.8 ± 4.5^{abc}	60 ± 16^{a}	2.2 ± 0.3^{c}	25.9 ± 6.8^{ab}
Less β-1,6-glucan						
kre6	10 ± 4^{cd}	2.3 ± 0.1^{cd}	4.5 ± 1.5^{def}	4 ± 3^{e}	2.1 ± 0.3^{c}	1.5 ± 1.1^{e}
Inner cell wall changes						
Less β-1,3-glucan						
knr4	0^{d}	–	0.0^{f}	0^{e}	–	0.0^{e}
fks1	1 ± 3^{cd}	2.0	0.5 ± 1.0^{f}	3 ± 3^{e}	2.2 ± 0.4^{bc}	1.1 ± 1.3^{e}
Less chitin						
chs3	19 ± 8^{bc}	1.6 ± 0.3^{e}	6.1 ± 2.5^{de}	28 ± 10^{bc}	1.5 ± 0.2^{d}	8.0 ± 3.0^{cd}
No changes						
WT	3 ± 3^{cd}	1.7 ± 0.1^{e}	0.9 ± 1.0^{ef}	0^{e}	–	0.0^{e}

Means were put together with the standard deviation (mean±S.D.). Each experiment was repeated twice: A and B. Values in the same column showing the same superscript letters are not significantly different ($p>0.05$). Strains were ordered according to their insufficiency on cell wall components (mannose, phosphomannose and glucans). Within each cell wall component, strains were organized by an increased proximity to the cell wall composition of the wild type strain in terms of mannose and glucans.

and 2) show that independently of the growth stage, the yeast genetic background can strongly influence *Artemia* performance. Compared with the WT yeast, total biomass production of nauplii was significantly improved when mnn10, mnn9, mnn8 and och1 were used as feed, due to both significant higher survival and/or individual length. These mutations interfere with the production of mannoproteins, by preventing α-1,6 mannose extensions (Fig. 1).

No differences or small significant differences were observed between nauplii fed WT yeast and nauplii fed mnn2, mnn5 and mnn1 yeast. In these mutants α-1,2 or α-1,3 branching of the mannose chain elongation is inhibited (Fig. 1).

Inhibition of the formation of phosphorylated mannoproteins on yeast (mnn4 and mnn6 mutants) resulted in significantly improved total biomass production of *Artemia*, especially with exp-phase harvested yeast cells. In these treatments, higher biomass production values were encountered due to considerable increases in survival.

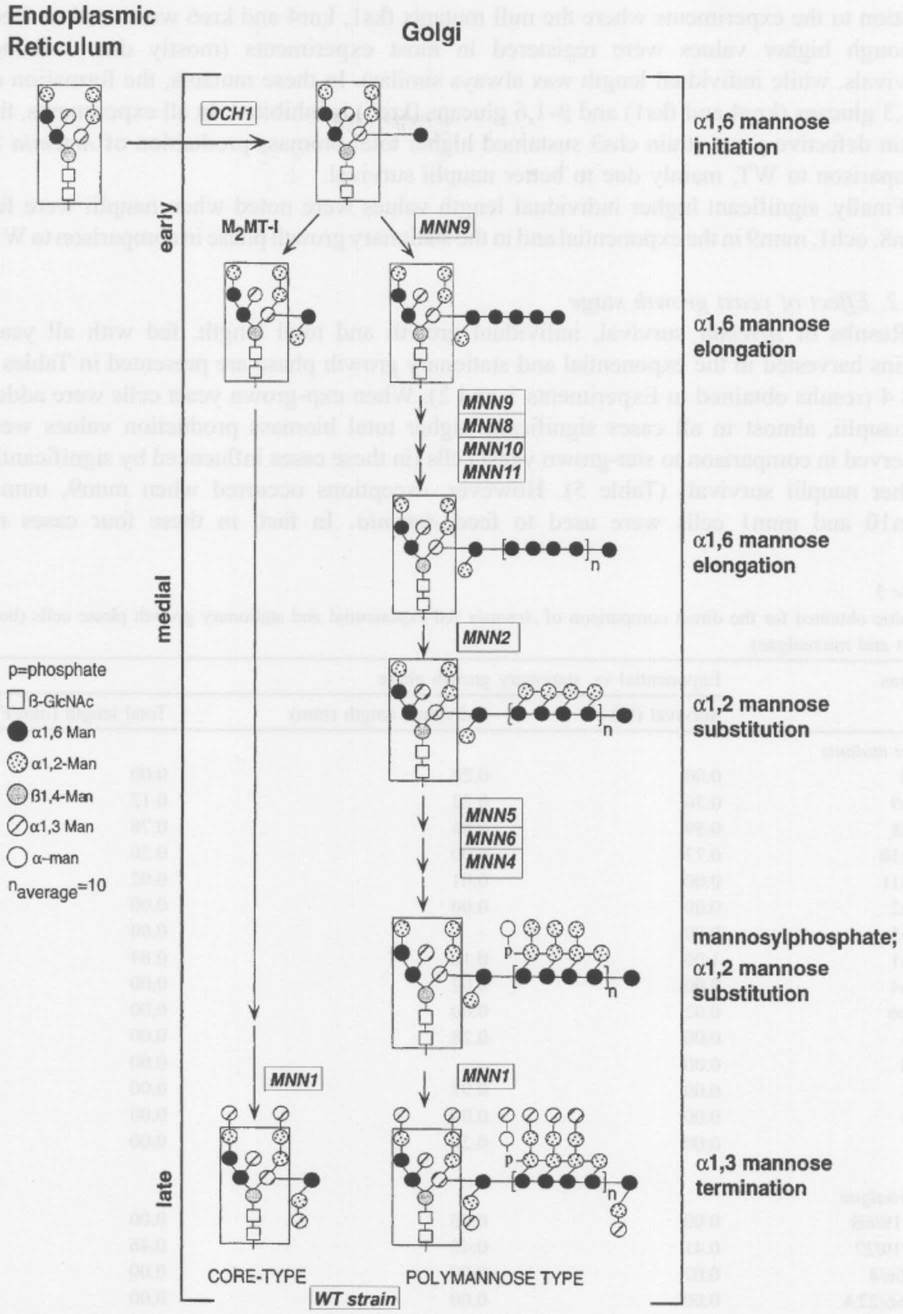

Fig. 1. A model for the structure and biosynthetic pathway of *S. cerevisiae* N-linked oligosaccharides in the Golgi complex in order to produce cell wall mannoproteins (adapted from Dean, 1999).

When fed WT yeast, no differences in total nauplii biomass production occurred in relation to the experiments where the null mutants fks1, knr4 and kre6 were used as feed, although higher values were registered in most experiments (mostly due to better survivals, while individual length was always similar). In these mutants, the formation of β-1,3 glucans (knr4 and fks1) and β-1,6 glucans (kre6) is inhibited. In all experiments, the chitin defective yeast strain chs3 sustained higher total biomass production of *Artemia* in comparison to WT, mainly due to better nauplii survival.

Finally, significant higher individual length values were noted when nauplii were fed mnn8, och1, mnn9 in the exponential and in the stationary growth phase in comparison to WT.

3.1.2. Effect of yeast growth stage

Results of *Artemia* survival, individual growth and total length, fed with all yeast strains harvested in the exponential and stationary growth phase are presented in Tables 3 and 4 (results obtained in Experiments 1 and 2). When exp-grown yeast cells were added to nauplii, almost in all cases significant higher total biomass production values were observed in comparison to stat-grown yeast cells (in these cases influenced by significantly higher nauplii survival) (Table 5). However, exceptions occurred when mnn9, mnn8, mnn10 and mnn1 cells were used to feed *Artemia*. In fact, in these four cases no

Table 5
p-value obtained for the direct comparison of *Artemia* fed exponential and stationary growth phase cells (both yeast and microalgae)

Strains	Exponential vs. stationary growth phase		
	Survival (%)	Individual length (mm)	Total length (mm/FT)
Yeast mutants			
och1	0.00	0.28	0.00
mnn9	0.36	0.22	0.12
mnn8	0.59	0.66	0.78
mnn10	0.73	0.00	0.26
mnn11	0.00	0.01	0.02
mnn2	0.00	0.00	0.00
mnn5	0.00	–	0.00
mnn1	1.00	0.16	0.84
mnn4	0.00	0.02	0.00
Mnn6	0.02	0.00	0.00
kre6	0.00	0.28	0.00
knr4	0.00	–	0.00
fks1	0.00	0.97	0.00
chs3	0.00	0.00	0.00
WT	0.00	0.20	0.00
Microalgae			
DT 19/6B	0.00	0.00	0.00
DT 19/27	0.41	0.45	0.46
TS 66/4	0.02	0.00	0.00
TS 66/22A	0.00	0.00	0.00

Three nauplii parameters were analyzed: survival (%), individual length (mm) and total length (mm per Falcon tube). Significant differences in nauplii performance were obtained when $p<0.05$.

significant differences were observed in total biomass production of nauplii (mostly due to both similar survivals and individual length).

As for individual length, the highest values were noted when nauplii were fed yeast harvested in the exponential phase. Yet, significant lower individual lengths were only observed when nauplii were fed with stat-grown mnn10, mnn2, mnn4, mnn6, mnn11 and chs3 cells.

3.2. *Artemia performance fed live microalgae cells*

3.2.1. *Effect of using different microalgae strains and species*

Two strains of axenically grown *D. tertiolecta* and *T. suecica* were tested as feed for *Artemia* (Table 6—results obtained in Experiment 3). In both exponential and stationary phase harvested cells, reproducible significant differences in total biomass production (total length) of *Artemia* were observed between the two strains of *D. tertiolecta* (in all four cases this was due to significant higher individual length when DT CCAP 19/6B was used, while only in two out of four cases survival was significantly higher).

In contrast, both *T. suecica* strains supported equally well the growth (total biomass production) of *Artemia*, due to similar nauplii survival and individual length obtained.

Furthermore, no significant differences in all three *Artemia* performance parameters were found between DT CCAP 19/6B and the two strains of *T. suecica*, while significantly poor nauplii results were observed when DT CCAP 19/27 was added as feed, in comparison to the other three microalgae strains.

Table 6
Experiment 3—average survival (%), individual length (mm) and total length (mm per Falcon tube—FT) of nauplii fed with two different strains of *D. tertiolecta* (DT CCAP 19/6B and DT CCAP 19/27) and two different strains of *T. suecica* (TS CCAP 66/4 and TS CCAP 66/22A) harvested in the exponential and in the stationary growth phases

Strains	A			B		
	Survival (%)	Individual length (mm)	Total length (mm/FT)	Survival (%)	Individual length (mm)	Total length (mm/FT)
Exponential growth phase						
DT 19/6B	98 ± 3^{a}	3.2 ± 0.7^{a}	67.5 ± 10.8^{a}	100^{a}	3.3 ± 0.6^{a}	72.3 ± 7.1^{a}
DT 19/27	68 ± 12^{b}	1.5 ± 0.3^{b}	20.9 ± 3.8^{b}	78 ± 13^{b}	1.6 ± 0.3^{b}	24.4 ± 4.2^{b}
TS 66/4	94 ± 6^{a}	3.5 ± 0.9^{a}	66.2 ± 5.6^{a}	94 ± 6^{ab}	3.7 ± 0.8^{a}	72.9 ± 8.8^{a}
TS 66/22A	99 ± 2^{a}	4.0 ± 0.8^{a}	88.3 ± 1.7^{a}	92 ± 3^{ab}	3.7 ± 0.6^{a}	67.5 ± 7.3^{a}
Stationary growth phase						
DT 19/6B	90 ± 9^{a}	2.7 ± 0.4^{b}	48.4 ± 4.8^{a}	81 ± 12^{a}	2.1 ± 0.3^{b}	34.3 ± 6.1^{a}
DT 19/27	77 ± 9^{a}	1.5 ± 0.2^{c}	24.3 ± 4.1^{b}	59 ± 3^{a}	1.6 ± 0.3^{c}	17.9 ± 0.9^{b}
TS 66/4	90 ± 7^{a}	2.8 ± 0.9^{b}	50.7 ± 9.1^{a}	51 ± 19^{a}	2.6 ± 1.0^{a}	26.7 ± 9.7^{a}
TS 66/22A	80 ± 7^{a}	3.1 ± 1.0^{a}	50.3 ± 5.7^{a}	55 ± 20^{a}	3.2 ± 1.1^{a}	36.1 ± 17.7^{a}

Means were put together with the standard deviation (mean±S.D.). Each experiment was repeated twice: A and B. Values in the same column (for each growth phase) showing the same superscript letter are not significantly different ($p > 0.05$). Strains were ordered according with their insufficiency on cell wall components (mannose, phosphomannose and glucans). Within each cell wall component, strains were organized by an increased proximity to the cell wall composition of the wild type strain in terms of mannose and glucans.

The DNA sequence analysis of both *D. tertiolecta* strains, using the nuclear rDNA internal transcribed spacer region 2, shows that both DT CCAP 19/6B (ASAP Genebank accession number AY572957) and DT CCAP 19/27 (ASAP Genebank accession number AY654300) are indeed a *D. tertiolecta*, since the sequence is almost identical to already published sequences of other *D. tertiolecta* strains, such as UTEX 999, CCMP 1320, and FHL.

3.3. Effect of microalgae growth stage

The comparison between *Artemia* performance fed with exp-grown and stat-grown microalgae strains is summarized in Tables 5 and 6 (results obtained in Experiment 3). When both strains of *T. suecica* and the *D. tertiolecta* strain DT CCAP 19/6B were used to feed *Artemia*, significantly higher total biomass production was noted with exp-grown cells, influenced by significantly higher nauplii survival and individual length. No significant differences were observed between nauplii fed with the other strain of *D. tertiolecta* DT CCAP 19/27 harvested in the exponential or in the stationary growth phase. This was due to similar nauplii survival and individual length.

3.4. Yeast and microalgae ash content and fatty acid composition

The cells of mnn9 seemed to have a higher AFDW content than the WT yeast cells (see Table 7) (although not statistically significant). This might have contributed to the considerable higher *Artemia* biomass production (total length) observed with mnn9. However, no significant difference in AFDW between exponential and stationary phase WT cells was observed, while significant different *Artemia* biomass occurred.

The individual AFDW of microalgae cells was significantly higher than for yeast cells. As the feeding regime with yeast and microalgae cells was considerably different, the disparity in AFDW input during the experimental period was less pronounced (Table 7). The higher AFDW of the microalgal cells is not believed to have contributed to the better performance of nauplii, since all treatments were fed to satiation. This was observed in all

Table 7
Average ash free dry weight (AFDW) of all microalgae strains, WT and mnn9 yeast cells harvested in the exponential growth phase, and stat-grown WT and mnn9 cells, expressed in mg/10^9 cells

Strains	AFDW (mg/10^9 cells)	AFDW (mg/Falcon tube)
WT exp	1.81 ± 0.36^{d}	0.19 ± 0.04^{d}
WT stat	1.56 ± 0.12^{d}	0.16 ± 0.01^{d}
mnn9 exp	3.52 ± 0.87^{d}	0.37 ± 0.09^{cd}
mnn9 stat	3.62 ± 0.20^{d}	0.38 ± 0.02^{cd}
DT 19/6B exp	40.52 ± 6.84^{c}	1.22 ± 0.21^{bc}
DT 19/27 exp	27.14 ± 19.84^{c}	0.81 ± 0.60^{cd}
TS 66/4 exp	184.49 ± 13.19^{a}	5.53 ± 0.40^{a}
TS 66/22A exp	93.46 ± 26.76^{b}	2.80 ± 0.80^{ab}

Values of AFDW are presented with the respective standard deviation (mean ± S.D.). WT—wild type strain; exp—exponential growth phase; stat—stationary growth phase. Values in the same column showing the same superscript letter are not significantly different ($p>0.05$).

Table 8
Fatty acid composition (% of total identified fatty acids) of the stationary grown (stat) WT yeast (WT) cultured in YEPD, the exponentially grown mnn9 cultured in YNB medium and the four microalgae strains harvested in the exponential growth phase (*D. tertiolecta*—DT CCAP 19/6B and DT CCAP 19/27—and *T. suecica*—TS CCAP 66/4 and TS CCAP 66/22A)

Fatty acid composition	WT stat YEPD	mnn9 exp YNB	DT CCAP 19/6B exp	DT CCAP 19/27 exp	TS CCAP 66/4 exp	TS CCAP 66/22A exp
Saturated						
12:0	0.5	0.5	0.5	0.6	0.6	0.1
13:0	nd	nd	1.1	1.5	1.0	0.9
14:0	0.6	0.8	0.9	1.1	1.4	1.0
15:0	0.1	0.5	0.8	1.0	0.8	0.6
16:0	14.0	19.8	17.0	15.5	18.6	20.5
17:0	tr	0.1	0.4	1.0	1.0	2.3
18:0	6.4	7.3	0.5	0.5	0.6	1.0
19:0	tr	nd	tr	tr	tr	0.1
20:0	tr	tr	0.1	tr	nd	tr
22:0	tr	0.1	nd	nd	nd	nd
24:0	nd	0.3	tr	tr	0.1	0.5
Sum	21.6	29.3	21.3	21.2	24.1	27.0
Monounsaturated						
14:1*n*-5	0.2	0.2	tr	tr	tr	tr
15:1*n*-5	0.2	0.3	0.6	0.7	0.4	0.3
16:1*n*-7	38.6	36.8	2.8	3.0	3.2	1.9
17:1*n*-7	0.1	0.1	nd	nd	tr	tr
18:1*n*-9	33.7	28.2	2.7	2.1	7.6	9.5
18:1*n*-7	3.1	2.7	1.4	1.6	3.2	0.5
19:1*n*-9	0.1	0.1	0.4	0.5	0.2	0.2
20:1*n*-9	tr	0.1	nd	tr	1.4	1.7
20:1*n*-7	nd	tr	nd	nd	tr	0.1
Sum	76.0	68.5	7.9	7.9	16.0	14.2
Polyunsaturated						
18:2*n*-6	0.6	0.5	4.1	3.8	5.0	10.1
18:3*n*-6	nd	nd	4.2	5.0	0.4	1.1
18:3*n*-3	0.1	0.1	40.0	39.0	16.3	9.6
20:3*n*-6	nd	nd	nd	nd	0.1	0.1
20:3*n*-3	nd	nd	0.1	0.1	0.2	0.1
Sum	0.7	0.6	48.4	47.9	22.0	21.0
Highly unsaturated						
16:4*n*-3	0.2	nd	15.1	16.2	15.5	11.3
18:4*n*-3	tr	0.1	2.0	2.2	9.5	9.9
20:4*n*-6	tr	nd	nd	nd	1.1	1.9
20:4*n*-3	nd	nd	nd	nd	0.8	0.7
20:5*n*-3	nd	nd	nd	tr	7.8	10.8
22:6*n*-3	nd	nd	nd	nd	nd	nd
Sum	0.2	0.1	17.1	18.4	34.7	34.6
Total FA	98.5	98.6	94.7	95.4	96.8	96.8
Total *n*-3	0.3	0.2	57.2	57.5	50.1	42.4
Total *n*-6	0.6	0.5	8.3	8.8	6.6	13.2
n-3:*n*-6	0.50	0.40	6.89	6.53	7.59	3.21

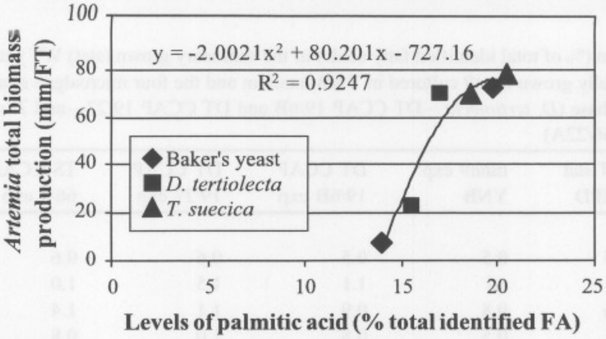

Fig. 2. Regression equation and respective r^2 obtained between the average total biomass production (mm/FT) of both experiments (A and B) and the levels of palmitic acid (16:0) expressed as a percentage of total identified fatty acids.

experiments as culture medium was clearly turbid by the end of the culture period. Significantly higher AFDW were obtained for the *T. suecica* strain TS CCAP 66/4 when compared to both *D. tertiolecta* strains, which was not reflected in total biomass production of nauplii fed with DT CCAP 19/6B.

Finally, no significant differences of AFDW were obtained between the two strains of *D. tertiolecta*, while *Artemia* performance with these strains was considerably different. Between the two strains of *T. suecica*, differences in the AFDW were observed, although not significant. In this case, no effect on *Artemia* performance was observed.

Microalgae and yeasts strains revealed similar relative amount of total saturated fatty acids, while considerable differences in unsaturated FA between the feeds were observed (see Table 8). In fact, for monounsaturated FA, yeasts showed the highest levels, whereas the opposite occurred for polyunsaturated and highly unsaturated FA. In between microalgal strains, both *T. suecica* strains presented the double amount of monounsaturated and highly unsaturated FA compared with *D. tertiolecta*, while they contained only half the relative amount of polyunsaturated FA. No clear-cut correlation between feed composition and *Artemia* performance was noticed for the six feeds mentioned in Table 8, except for palmitic acid (16:0) (Fig. 2).

4. Discussion

According to the results obtained in the present study, the genetic background of yeast has a strong influence on *Artemia* performance. The biochemical pathway for the synthesis of cell wall mannoproteins in *S. cerevisiae* is schematized in Fig. 1. The assignment of the step at which each candidate mannosyltransferase gene acts is based both on its mutant phenotype and/or on characterisation of its protein product (Munro, 2001).

Synthesis of cell wall mannoproteins starts in the endoplasmic reticulum, where the *N*-glycans oligosaccharides (backbone of mannoproteins) are produced. After transportation to the Golgi complex, which is the principal site within a cell for the terminal carbohydrate modification of proteins and lipids, the N-linked oligosaccharides are modified progressively and sequentially by addition of mannoses (Dean, 1999). Initially, all *N*-glycans receive a single α-1,6-mannose from the OCH1 protein transferase (encoded by the OCH1 gene). This mannose then serves as the scaffold for the further extension of the outer chain. Two related enzymes or enzyme complexes allow the extension of the mannan backbone by adding more α-1,6-mannose: the first include the MNN9 gene product, and the second comprises also the MNN9 gene product, as well as the MNN8, MNN10 and MNN11 gene products. The branches on the mannan backbone are initiated and then extended by two α-1,2-mannosyltransferases encoded by MNN2 and MNN5. Finally, proteins encoded by MNN6 and MNN4 add the mannose phosphates, and the terminal α-1,3-linked mannose is encoded by the MNN1 gene (Wills et al., 2000). After the MNN1 step, cell wall proteins similar to proteins present in the yeast cell wall of the wild type strain are formed. Some mannoproteins can contain up to 200 mannose units and several mannosylphosphate residues that endow the oligosaccharide with a negative charge (Dean, 1999), changing the properties and environment of the cell surface (Jigami and Odani, 1999). A mutation in one of the previous genes allows the addition of mannoses before the step in which the mutated gene should work, but prevents further elongation of the mannoprotein backbone (Dean, 1999). In general, these null mutants exhibit phenotypes characteristic of defects in cell wall biosynthesis and/or assembly, including poor cell wall growth, cell clumping and osmotic sensitivity (Cid et al., 1995; Jungmann et al., 1999).

Since all mnn strains display reduced concentrations of mannose structures present in the cell wall in comparison to the WT strain, yeast compensates this deficiency by increasing the amounts of the other two cell wall constituents (chitin and glucans) (Magnelli et al., 2002) in order to avoid cell lysis (Klis et al., 2002).

In the present study, yeast strains containing low concentrations of mannoproteins in the cell wall, always supported a high total biomass production of *Artemia* nauplii. This was the case for mnn9, mnn10, mnn8 and och1 mutants. In addition, independently of the growth phase, these mutants always gave the highest nauplii growth. According to Jigami and Odani (1999), mannoproteins located in the outermost 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. Furthermore, as reported by 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 to yeast digestion by the meta-nauplii. Therefore, it is likely that digestive enzymes of *Artemia* (such as β-glucanase) can easily enter and contribute to the proper digestion of yeast cells with reduced mannoprotein content.

Similar results were obtained by Marques et al. (2004) when comparing the performance of nauplii fed with mnn9 and WT yeast strains cultured under different conditions. A strong correlation between yeast cell mannoprotein content and *Artemia* performance was observed in the previous study. On the basis of that study, *Artemia* fed with yeast strains defective in β-glucan synthesis (kre6, knr4 and fks1) and chitin synthesis (chs3) were expected to show lower performance, compared to nauplii fed WT yeast, due to the higher levels of mannan present in the cell wall of these four mutants (according to Dallies et al., 1998; Magnelli et al., 2002; Pagé et al., 2003). However, in the present study, improved *Artemia* performance was obtained with yeast mutants with reduced β-1,3 glucans (knr4 and fks1), β-1,6 glucans (kre6) and chitin (chs3) levels (not always significantly). These results seem to indicate that the levels of mannan present in the yeast cell wall are not the only factor influencing nauplii performance.

According to Aguillar-Uscanga and François (2003), β-1,3 and especially β-1,6 glucans provide anchorage to most cell wall mannoproteins and are also covalently associated with chitin, contributing to the modular structure of the cell wall. β-1,3 glucans also provide the rigidity and integrity of the cell wall, and determines the cell shape (Martin-Yken et al., 2002). As a result, lack of β-glucans in the yeast cell wall might result in less covalent linkages between the three cell wall compounds, contributing to a more permeable cell wall in comparison to the WT strain. Similarly, higher nauplii performance occurred when the chs3 yeast strain (chitin defective) was used to feed *Artemia* in comparison to the WT strain. Although chitin concentration in the yeast cell wall corresponds only to 1–2% of the cell wall dry mass in a wild type strain, this compound is an essential element for the yeast cell wall (Magnelli et al., 2002). In fact, chitin plays a key role in yeast cell growth and division (Cabib et al., 2001), as well as in its morphogenesis, and is essential for the viability of yeast and fungal cells (Valdivieso et al., 2000), contributing to the strength of the cell wall (Klis et al., 2002). Since chitin is attached covalently to β-1,3 glucans, β-1,6 glucans and mannoproteins (Cabib et al., 2001), the unexpected results obtained with nauplii fed chitin defective yeast could also be due to an improved digestibility of chs3-cells by *Artemia*, caused by the reduced linkage between the three cell wall compounds.

In conclusion, it seems that the density of covalent linkages between the three yeast cell wall constituents plays an important role in the enhancement of yeast digestibility by *Artemia*, as well as high amounts of cell wall bound chitin and glucans in combination with low amounts of mannoproteins.

Artemia performance tended to be higher when exponentially grown yeast strains were used as feed, in comparison to the same yeast strains harvested in the stationary growth phase. The differences were more pronounced in nauplii survival than in individual growth. Our results are in accordance with previous results obtained by Coutteau et al. (1990) for xenic baker's yeast, who reported higher *Artemia* survival and growth when fed wild type yeast harvested in the exponential growth phase (averages of 52.2% survival and 2.93 mm growth after 8 days) in comparison to nauplii fed stat-grown yeast (averages of 13.3% survival and 2.11 mm growth after 8 days). Marques et al. (2004) obtained similar results with nauplii fed mnn9 and wild type yeast cells under gnotobiotic conditions. According to Klis et al. (2002), yeast cells entering in stationary phase, form different walls, becoming 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 (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 (Deutch and Parry, 1974; De Nobel et al., 2000; Cabib et al., 2001). The phosphomannan complexes present in the outer yeast cell wall and the presence of disulphide bridges work as a barrier affecting the penetration of the digestive/degradative enzymes to access the glucan complexes (present in the inner yeast cell wall) and the yeast cell content (Killick, 1971). However, when exp-grown mnn9, mnn8 and mnn10 were added as feed, nauplii results were similar to stat-grown yeasts, which could be a consequence of the reduced possibility for cross-linking mannoproteins in the cell wall.

Yeast strains with reduced phosphomannan levels in the cell wall (especially mnn6) gave always higher *Artemia* performance (especially on survival) when compared to nauplii fed WT yeast. This fact could be due to the likely implication of phosphomannans in phosphodiester cross-linking of mannoproteins to β-glucans (Jigami and Odani, 1999).

Yeast digestibility by *Artemia* can thus be significantly improved by manipulating the genetic background, growth stage and medium used (Marques et al., 2004). According to the previous authors, when cultured in particular conditions, yeast can sustain optimal nauplii performance comparable to results obtained with microalgae (e.g. exp-grown mnn9 cultured in YNB).

Artemia performance using axenic *D. tertiolecta* and *T. suecica* cells as feed was very good (Table 6). The obtained results were similar to those found by Sick (1976), for axenic *Artemia* fed sterile *D. tertiolecta* (3 mm growth after 6 days), and by Coutteau et al. (1990) for xenic nauplii fed with the same microalga (averages of 94.3% survival and 4.22 mm growth after 8 days). Results reported by Walne (1967) for xenic *Artemia* fed with *T. suecica* were lower than in our study (at day 4, nauplii had just 1.3 mm length). In contrast, Fábregas et al. (1996) and Thinh et al. (1999) obtained similar results when feeding xenic *Artemia* with *T. suecica* (respectively: 85% survival and 8.3 mm after 19 days; and 65–97% survival after 7 days).

In the present study, *Artemia* performance was dependent on the *D. tertiolecta* strain used, but not on the *T. suecica* strain (only for the strains that were tested, maybe others give differences). According to Orcutt and Patterson (1974), Enright et al. (1986), Guckert and Cooksey (1990), Herrero et al. (1991), Fábregas et al. (1995, 1996, 2001), Brown et al. (1997), Otero and Fábregas (1997) and D'Souza et al. (2000), the nutritional value of microalgae can vary significantly between species and culture conditions, such as microalgae concentration, method and duration of storage, pH, salinity, light flux, temperature, nutrient concentration and nutrient composition. The present study indicates that also the strain of *D. tertiolecta* and the growth stage of both microalgae species used to feed *Artemia* have a strong influence on the nutritional value of these microalgae to nauplii. In fact, exp-grown microalgae strains sustained significantly better nauplii performance (especially for DT CCAP 19/6B, TS CCAP 66/4 and TS CCAP 66/22A) than stat-grown microalgae cells (Table 5). According to Webb and Chu (1983) and Dunstan et al. (1993), microalgae cells in the exponential phase may differ in nutritional composition to those in the stationary phase. Microalgae grown to late-logarithmic growth phase typically contain 30% to 40% proteins, 10% to 20% lipids and 5% to 15% carbohydrates (Brown et al., 1997; Renaud et al., 1999), while stat-grown microalgae can double carbohydrate levels at the expense of proteins (Harrison et al., 1990; Brown et al., 1993). In addition, microalgae are usually rich in polar lipids during exponential phase and accumulate triacylglycerol during stationary phase (Dunstan et al., 1993). According to D'Agostino (1980), lipids, as well as proteins, are the main nutrients required during the early developmental stages of *Artemia*, whereas carbohydrates, together with proteins, are more important for juveniles and adults. Therefore, the relative proportion of these three components should be considered with respect to optimal nauplii growth. In fact, higher lipid content and lower carbohydrate-to-lipid ratio in the microalga *T. suecica* improved *Artemia* survival and growth (Fábregas et al., 2001). Since the present study was performed with early developmental stages and juveniles of *Artemia*, the fatty acid composition of all microalgae strains and baker's yeast was determined (see Table 8). Our results of FA composition are in accordance with previous experiments performed by Delaunay et al. (1993), Zhukova and Aizdaicher (1995), Brown et al. (1997), Caers et al. (1998) and Nevejan et al. (2003) for *D. tertiolecta*, by Zhukova and Aizdaicher (1995), Brown et al. (1997), Caers et al. (1998) and Fábregas et al. (2001) for *T. suecica* and by Torija et al. (2003) for baker's yeast *S. cerevisiae*. As expected, in terms of aquaculture purposes, low nutritional quality of FA composition occurred in *D. tertiolecta* and baker's yeast (containing very few fatty acids longer or more unsaturated than linolenic acid—18:3), while high quality fatty acids were present in *T. suecica* (Brown et al., 1997; Caers et al., 1998). Marine larvae, in general, require polyunsaturated fatty acids, such as eicosapentaenoic acid EPA (20:5*n*-3) and docosahexaenoic acid DHA (22:6*n*-3), for their normal development and survival (Navarro et al., 1999). However, in the present study, *Artemia* performance was similar for DT CCAP 19/6B and both strains of *T. suecica*. Therefore, it seemed that *n*-3 HUFA were not essential nutrients for *Artemia*, as previously reported by D'Agostino (1980). Furthermore, no major differences were observed between the fatty acid composition of the two strains of *D. tertiolecta*, as well as for its AFDW, while nauplii performances were significantly different. However, levels of palmitic acid (16:0) present in all feeds were highly correlated with the average total biomass production of *Artemia* for both experiments (A and B) (r=0.962, p=0.0004, n=6). According to Provasoli and Pintner (1980) and D'Agostino (1980), levels of myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) fatty acids seem to stimulate growth and fertility of *Artemia* when added to the culture medium. Hence, the low nutritional value of the strain DT CCAP 19/27 could be attributed to levels of specific fatty acids, but also other undetermined factors could interfere, such as protein content, micronutrients and digestibility as it is the case for yeast strains.

5. Conclusions

The 14 yeast mutants constitute an interesting collection of feed sources to modulate the growth performance of *Artemia* under gnotobiotic conditions. The results suggest that any weakening of the three-dimensional structure of the yeast cell wall has a positive effect in its survival-associated nutritional value for *Artemia* when the latter is grown under gnotobiotic or axenic conditions. This weakening can be obtained by reducing the mannoprotein, the chitin or the β-glucan content of the cell wall. Reducing the mannoprotein content (e.g. MNN9 mutation) seems to be the most positive effect as the *Artemia* growth performance is high and less dependent on the yeast growth stage. Simply avoiding phosphorylation of mannoproteins is another way of increasing yeast digestibility. Axenic microalgal strains differ also in their nutritional value for bacteria-free grown *Artemia*, be it in a less defined way. It is anticipated that the developed gnotobiotic *Artemia* growth tests will constitute excellent tools for the study of bacterial effects in aquatic food webs. In fact, many questions remain unanswered regarding the precise mechanism of action of specific probiotics and pathogens associated to aquatic organisms. The combination of gnotobiotic cultures and other techniques (e.g. genetics expression analysis in the host and histology facilitated by the use of FISH analysis) should provide a better understanding of several issues, such as: host–microbe interactions (e.g. stimulation of host immune response), microbial–microbial interactions (e.g. competition for adhesion sites, chemicals, available energy or iron; production of inhibitory compounds; dynamic of the population in the gastrointestinal tract) and water quality. Such understandings, in turn, could lead to the development of novel chemicals and microbes for use in prebiotic and probiotic strategies to prevent or cure diseases.

Acknowledgments

The Foundation for Science and Technology (FCT) of Portugal supported this study through a doctoral grant to the first author (no. SFRH/BD/4831/2001). We thank the Belgian Foundation for Scientific Research (FWO) for financing the project “Functional role and characteristics of micro-organisms in the larviculture of aquatic organisms: *Artemia* as preferred test organism” (no. 350230.02) and the project “Nutritional and immunostimulatory characteristics of isogenic yeast mutants in *Artemia*” (no. 1.5.125.04). The technical assistance of the staff of the chemical laboratory at the Artemia Reference Center for the FAME analyses is greatly appreciated. We are also grateful to Juergen Polle from the Department of Biology of the Brooklyn College, USA, for the DNA sequencing and verification of the two *D. tertiolecta* strains used in this study, and to Kristof Dierckens, Els Vanden Berghe and Bart Van Delsen of the Artemia Reference Center for the revision and comments. **[SS]**

References

Aguilar-Uscanga, B., François, J., 2003. A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation. Lett. Appl. Microbiol. 37, 268–274.

Brown, M., Garland, C., Jeffrey, S., Jameson, I., Leroi, J., 1993. The gross and amino acid compositions of batch and semi-continuous cultures of *Isochrysis* sp. (clone T.ISO), *Pavlova lutheri* and *Nannochloropsis oculata*. J. Appl. Phycol. 5, 285–296.

Brown, M., Jeffrey, S., Volkman, J., Dunstan, G., 1997. Nutritional properties of microalgae for mariculture. Aquaculture 151, 315–331.

Cabib, E., Roh, D., Schmidt, M., Crotti, L., Varma, A., 2001. The yeast cell wall and septum as paradigms of cell growth and morphogenesis—mini review. J. Biol. Chem. 276 (23), 19679–19682.

Caers, M., Coutteau, P., Lombeida, P., Sorgeloos, P., 1998. The effect of lipid supplementation on the growth and fatty acid composition of *Tapes philippinarum* (L.) spat. Aquaculture 162, 287–299.

Cid, V., Duran, A., Del Rey, F., Snyder, M., Nombela, C., Sanchez, M., 1995. Molecular basis of cell integrity and morphogenesis in *Saccharomyces cerevisiae*. Microbiol. Rev. 59 (3), 345–386.

Coutteau, P., Sorgeloos, P., 1995. Intercalibration exercise on the qualitative and quantitative analysis of fatty acids in *Artemia* and marine sample used in mariculture. ICES Coop. Res. Rep. 211, 1–30.

Coutteau, P., Lavens, P., Sorgeloos, P., 1990. Baker’s yeast as a potential substitute for live algae in aquaculture diets: artemia as a case study. J. World Aquac. Soc. 21 (1), 1–8.

D'Agostino, A., 1980. The vital requirements of *Artemia*: physiology and nutrition. In: Persoone, G., Sorgeloos, P., Roels, O., Jaspers, R. (Eds.), The Brine Shrimp Artemia. Physiology, Biochemistry, Molecular Biology, vol. 2. Universa Press, Wetteren, Belgium, pp. 55–82.

Dallies, N., François, J., Paquet, V., 1998. A new method for quantitative determination of polysaccharides in the yeast cell wall. Application to cell wall defective mutants of *Saccharomyces cerevisiae*. Yeast 14, 1206–1397.

Dean, N., 1999. Asparagine-linked glycosylation in the yeast Golgi (review). Biochim. Biophys. Acta 1426, 309–322.

De Groot, P., Ruiz, C., Aldana, C., Dueñas, E., Cid, V., Del Rey, F., Rodríguez-peña, J., Pérez, P., Andel, A., Caubin, J., Arroyo, J., Garcia, J., Gil, C., Molina, M., García, L., Nombela, C., Klis, F., 2001. A genomic approach for the identification and classification of genes involved in cell wall formation and its regulation in *Saccharomyces cerevisiae*. Compar. Funct. Genom. 2, 124–142.

Delaunay, F., Marty, Y., Moal, J., Samain, J., 1993. The effect of monospecific algal diets on growth and fatty acid composition of *Pecten maximus* (L.) larvae. J. Exp. Mar. Biol. Ecol. 173, 163–179.

De Nobel, H., Ruiz, C., Martin, H., Morris, W., Brul, S., Molina, M., Klis, F., 2000. Cell wall perturbation in yeast results in dual phosphorylation of the Slt2/Mpk1 MAP kinase and in an Slt2-mediated increase in FKS2-lacZ expression, glucanase resistance and thermo tolerance. Microbiology 146, 2121–2132.

Deutch, C., Parry, J., 1974. Sphaeroplast formation in yeast during the transition from exponential to stationary phase. J. Gen. Microbiol. 80, 259–268.

Dobbeleir, J., Adam, N., Bossuyt, E., Bruggeman, E., Sorgeloos, P., 1980. New aspects of the use of inert diets for high density culturing of brine shrimp. In: Persoone, G., Sorgeloos, P., Roels, O., Jasper, E. (Eds.), The Brine Shrimp *Artemia*. Ecology, Culture, Use in Aquaculture, vol. 3. Universa Press, Wetteren, Belgium, pp. 165–174.

D'Souza, F., Lecossois, D., Heasman, M., Diemar, J., Jackson, C., Pendrey, R., 2000. Evaluation of centrifuged microalgae concentrates as diets for *Penaeus monodon* (Fabricius) larvae. Aquac. Res. 31, 661–670.

Dunstan, G., Volkman, J., Barrett, S., Leroi, J., Jeffrey, S., 1993. Changes in the lipid composition and maximization of the polyunsaturated fatty acid content of three microalgae grown in mass culture. J. Appl. Phycol. 5, 71–83.

Enright, C., Newkirk, G., Craigie, J., Castell, J., 1986. Growth of juvenile *Ostrea edulis* L. fed *Chaetoceros calcitrans* Schütt of varied chemical composition. J. Exp. Mar. Biol. Ecol. 96, 15–26.

Fábregas, J., Patino, M., Vecino, E., Cházaro, F., Otero, A., 1995. Productivity and biochemical composition of cyclostat cultures of the marine microalga *Tetraselmis suecica*. Appl. Microbiol. Biotechnol. 43, 617–621.

Fábregas, J., Otero, A., Morales, E., Cordero, B., Patiño, M., 1996. *Tetraselmis suecica* cultured in different nutrient concentrations varies in nutritional value to *Artemia*. Aquaculture 143, 197–204.

Fábregas, J., Otero, A., Domínguez, A., Patiño, M., 2001. Growth rate of the microalga *Tetraselmis suecica* changes the biochemical composition of *Artemia* species. Mar. Biotechnol. 3, 256–263.

Gorospe, J., Nakamura, K., Abe, M., Higashi, S., 1996. Nutritional contribution of *Pseudomonas* sp. in *Artemia* culture. Fish. Sci. 62, 914–918.

Guckert, J., Cooksey, K., 1990. Triglyceride accumulation and fatty acid profile changes in *Chlorella* (Chlorophyta) during high pH-induced cell cycle inhibition. J. Phycol. 26, 72–79.

Harrison, P., Thompson, P., Calderwood, G., 1990. Effects of nutrient and light limitation on the biochemical composition of phytoplankton. J. Appl. Phycol. 2, 45–56.

Herrero, C., Cid, A., Fábregas, J., Abalde, J., 1991. Yields in biomass and chemical constituents of four commercially important marine microalgae with different culture media. Aquac. Eng. 10, 99–110.

Intriago, P., Jones, D., 1993. Bacteria as food for *Artemia*. Aquaculture 113, 115–127.

Jigami, Y., Odani, T., 1999. Mannosylphosphate transfer to yeast mannan (review). Biochim. Biophys. Acta 1426, 335–345.

Johnson, D., 1980. Evaluation of various diets for optimal growth and survival of selected life stages of Artemia. In: Persoone, G., Sorgeloos, P., Roels, O., Jaspers, E. (Eds.), The Brine Shrimp Artemia. Ecology, Culturing, Use in Aquaculture, vol. 3. Universa Press, Wetteren, Belgium, pp. 185–192.

Jungmann, J., Rayner, J., Munro, S., 1999. The *Saccharomyces cerevisiae* protein MNN10p/Bed1p is a subunit of a Golgi mannosyltransferase complex. J. Biol. Chem. 274 (10), 6579–6585.

Killick, K., 1971. Modification of the composition and structure of the yeast cell wall by culture in the presence of sulfur amino acids. J. Bacteriol. 106 (3), 931–937.

Klis, K., Mol, P., Hellingwerf, K., Brul, S., 2002. Dynamics of cell wall structure in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 26, 239–256.

Lagorce, A., Hauser, N., Labourdette, D., Rodriguez, C., Martin-Yken, H., Arroyo, J., Hoheisel, J., François, J., 2003. Genome-wide analysis of the response to cell wall mutations in the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. 278 (22), 20345–20357.

Magnelli, P., Cipollo, J., Abeijon, C., 2002. A refined method for the determination of *Saccharomyces cerevisiae* cell wall composition and b-1,6-glucan fine structure. Anal. Biochem. 301, 136–150.

Marques, A., Bossier, P., Dhont, J., Sorgeloos, P., 2004. Influence of yeast quality on performance of gnotobiotically-grown *Artemia*. J. Exp. Mar. Biol. Ecol. 310 (2), 249–266.

Martin-Yken, H., Lagorce, A., Dagkessamanskaia, A., François, J., 2002. Yeast cell wall structure and assembly in relation with cell growth and morphogenesis. Recent Res. Dev. Biochem. 6, 503–527.

Munro, S., 2001. What can yeast tell us about N-linked glycosilation in the Golgi apparatus (Minireview). FEBS Lett. 498, 223–227.

Navarro, J., Henderson, R., McEvoy, L., Bell, M., Amat, F., 1999. Lipid conversions during enrichment of *Artemia*. Aquaculture 174, 155–166.

Nevejan, N., Saez, I., Gajardo, G., Sorgeloos, P., 2003. Supplementation of EPA and DHA emulsions to a *Dunaliella tertiolecta* diet: effect on growth and lipid composition of scallop larvae, *Argopecten purpuratus* (Lamarck, 1819). Aquaculture 217, 613–632.

Odani, T., Shimma, Y., Wang, X., Jigami, Y., 1997. Mannosylphosphate transfer to cell wall mannan is regulated by the transcriptional level of the MNN4 gene in *Saccharomyces cerevisiae*. FEBS Lett. 420 (2–3), 186–190.

Orcutt, D., Patterson, G., 1974. Effect of light-intensity upon lipid-composition of *Nitzschia closterium* (Cylindrotheca—Fusiformis). Lipids 9 (12), 1000–1003.

Otero, A., Fábregas, J., 1997. Changes in the nutrient composition of *T. suecica* cultured semicontinuously with different nutrient concentrations and renewal rates. Aquaculture 159, 111–123.

Pagé, N., Gérard-Vincent, M., Ménard, P., Beaulieu, M., Azuma, M., Dijkgraaf, G., Li, H., Marcoux, J., Nguyen, T., Dowse, T., Sdicu, A., Bussey, H., 2003. A *Saccharomyces cerevisiae* genome-wide mutant screen for altered sensitivity to K1 killer toxin. Genetics 163, 875–894.

Provasoli, L., Pintner, I., 1980. Biphasic particulate media for the parthenogenetic *Artemia* of Sète. In: Persoone, G., Sorgeloos, P., Roels, O., Jaspers, R. (Eds.), The Brine Shrimp Artemia. Physiology, Biochemistry, Molecular Biology, vol. 2. Universa Press, Wetteren, Belgium, pp. 231–238.

Renaud, S., Thinh, L., Parry, D., 1999. The gross composition and fatty acid composition of 18 species of tropical Australian microalgae for possible use in mariculture. Aquaculture 170, 147–159.

Sick, L., 1976. Nutritional effect of five species of microalgae on the growth, development and survival of the brine shrimp *Artemia salina*. Mar. Biol. 35 (1), 69–78.

Sladowski, D., Steer, S., Clothier, R., Balls, M., 1993. An improved MTT assay. J. Immunol. Methods 157, 203–207.

Sorgeloos, P., Lavens, P., Léger, P., Tackaert, W., Versichele, D., 1986. Manual for the Culture and Use of Brine Shrimp *Artemia* in Aquaculture. Artemia Reference Center, Faculty of Agriculture, State University of Ghent, Belgium, p. 318.

Sorgeloos, P., Dhert, P., Candreva, P., 2001. Use of brine shrimp, *Artemia* sp., in marine fish larviculture. Aquaculture 200, 147–159.

Thinh, L., Renaud, S., Parry, D., 1999. Evaluation of recently isolated Australian tropical microalgae for the enrichment of the dietary value of brine shrimp, *Artemia nauplii*. Aquaculture 170 (2), 161–173.

Torija, M., Beltran, G., Novo, M., Poblet, M., Rozés, N., Guillamón, J., Mas, A., 2003. Effect of the nitrogen source on the fatty acid composition of *Saccharomyces cerevisiae* (Research Note). Food Microbiol. 20, 255–258.

Valdivieso, M., Ferrario, L., Vai, M., Duran, A., Popolo, L., 2000. Chitin synthesis in a gas1 mutant of *Saccharomyces cerevisiae*. J. Bacteriol. 182 (17), 4752–4757.

Verdonck, L., Swings, J., Kersters, K., Dehasque, M., Sorgeloos, P., Leger, P., 1994. Variability of the microbial environment of rotifer *Brachionus plicatilis* and *Artemia*. J. World Aquac. Soc. 25 (1), 55–59.

Walne, P., 1967. The food value of 13 species of unicellular algae to *Artemia salina*. ICES CM:1967, vol. E:5. Academic Press, Denmark, p. 6.

Webb, K., Chu, F., 1983. Phytoplankton as a food source for bivalve larvae. In: Pruder, G., Langdon, C., Conklin, D. (Eds.), Proceedings of the Second International Conference on Aquaculture Nutrition: Biochemical and Physiological Approaches to Shellfish Nutrition. Louisiana State University, Baton Rouge, LA, pp. 272–291.

Wills, E., Redinbo, M., Perfect, J., Del Poeta, M., 2000. Anti-infectives: new potential targets for antifungal development (review). Expert Opin. Ther. Targets 4 (3), 1–32.

Zhukova, N., Aizdaicher, N., 1995. Fatty acid composition of 15 species of marine microalgae. Phytochemistry 39 (2), 351–356.