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Nutritional value of six Pavlovophyceae for Crassostrea gigas and Pecten maximus larvae

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Abstract: Four unidentified new strains of Pavlovophyceae [Pavlova sp. AC 250 (Pfl), Pavlova sp. AC 248 (Psh), Pavlova sp. AC 251 (Pth), Pavlova sp. AC 538 (Psm)] and two known species [Pavlova pinguis (Ppi), Rebecca salina (ex Pavlova salina, Rsa)] were characterized (productivity, size, dry weight, ash, gross composition, fatty acids, sterols) and their nutritional value in bispecific diets were evaluated both on Crassostrea gigas and Pecten maximus larvae. All microalgae exhibited poor food value for C. gigas larvae. P. pinguis and R. salina did not sustain any growth, like the control starved larvae. No exo-toxicity of P. pinguis and R. salina was detected. These two species were weakly ingested by C. gigas larvae, while the four other pavlovophytes were effectively grazed. When used as food for P. maximus larvae, P. pinguis and Psh led to poor development. In contrast, a diet with Pfl resulted in significantly better growth than the control.

Keywords: Microalgae; Nutritional value; Larval nutrition; Biochemical composition

1. Introduction

In mollusc hatcheries the main criteria for the suitability of microalgal strains are that they should be readily and easily cultured, rapidly ingested and digested, and they must exhibit a high nutritional value, particularly with respect to the highly unsaturated fatty acids (Robert et al., 2004). Less than ten microalgal strains are routinely used in mollusc hatcheries, and this fact represents a constraint for the full development of this activity (Coutteau and Sorgeloos, 1992; Robert et al., 2004). Accordingly, the identification of new microalgal species of good food value for larvae constitutes one of the priorities of research related to this field (Brown et al., 1998; Renaud et al., 1999; Knuckey et al., 2002; Renaud et al., 2002). The microalgae routinely produced as feed in commercial mollusc hatcheries includes several diatoms (*Skeletonema costatum, Thalassiosira pseudonana* clone 3H, *Chaetoceros gracilis, C. calcitrans, C. calcitrans forma pumilum, C. muelleri*) and only two flagellates (*Isochrysis* aff. *galbana* clone T-Iso, *Pavlova lutheri*), while *Tetraselmis sp.* is somewhat used mainly for spat.
The haptophyte class Pavlovophyceae is composed by flagellates of appropriate size (3-5 µm) for ingestion by mollusc larvae. These species are also generally rich in polyunsaturated fatty acids (PUFA), which are essentials for molluscs (Volkman et al., 1991; Kanazawa et al., 1979). Different pavlovophytes have been used as feeds for molluscs (*Pavlova lutheri*, *P. pinguis*, *Rebecca salina*) with contrasting outcomes, depending on the mollusc species and on the stage concerned: *P. lutheri* was found to be effective for feeding *Pecten maximus* and *P. fumatus* larvae, while it was of poor food value for *C. gigas larvae* (Heasman et al., 2000; Ponis et al., 2003a,b; Laing, 2004); *P. pinguis* was successfully used as food for *C. gigas* spat (Brown et al., 1998; McCausland et al., 1999); *R. salina* has been used to feed different types of oysters (*C. gigas* spat, McCausland et al., 1999; *Pinctada margaritifera* larvae, Doroudi et al., 1999, 2003; Southgate et al., 1998; *Saccostrea echinata* larvae, Southgate and Lee, 1998).
In this study, six pavlovophytes were characterized in terms of cell density, productivity, dry weight, ash content, gross composition, and fatty acid and sterol contents in order to assess their potential food value for larval nutrition. These species were then tested as food for Pacific oyster (*C. gigas*) and king scallop (*P. maximus*) larvae.

2. Materials and methods

2.1 Microalgal culture

The six strains were selected from the Algobank-Caen microalgal culture collection of the University of Caen Basse-Normandie. All strains were clonal, a single cell having been isolated from seawater samples (from diverse locations) by micropipette (Table 1). They will be quoted in the text as Pfl = *Pavlova sp.* AC 250, Psh = *Pavlova sp.* AC248, Pth = *Pavlova sp.* AC 87, Psm = *Pavlova sp.* AC 538, Ppi = *Pavlova pinguis* and Rsa = *Rebecca salina* (*ex Pavlova salina*). Microalgae were grown in 2-l glass carboys at 22-23 °C under continuous illumination at an intensity of 180-220 µmol photons m^{-2} s^{-1}, provided by cool white fluorescent tubes. Seawater (salinity 34-35‰) was 1 µm filtered, enriched with sterilized Conway medium (Walne, 1966) and then autoclaved. A 3% CO_2-air mixture was supplied in order to support growth and to maintain the pH within a range of 7.5-8.1. For culture of the diatom *Chaetoceros calcitrans* forma *pumilum*, sodium metasilicate (40 mg l^{-1}) was added as a silica source and salinity was reduced to 25‰ by addition of distilled water.

Table 1. Size and growth characteristics of the six pavlovophytes used in the experimental feeding trials (mean ± S.D.; size n ≥ 15000; concentration and growth rate n = 2).

Strains	Code	Origin	Diameter (µm)	Concentration ($x10^6$ ml^{-1})	Growth rate (division day^{-1})
Pavlova sp. **(**Pfl**)**	(AC 250)	Mediterranean	5.3 ± 1.5	9.7 ± 0.7	0.7 ± 0.1
Pavlova sp. **(**Psh**)**	(AC248)	Mediterranean	4.5 ± 0.6	12.7 ± 0.5	0.8 ± 0.1
Pavlova sp. **(**Psm**)**	(AC 538)	North Atlantic	4.7 ± 0.9	13.4 ± 0.8	0.8 ± 0.1
Pavlova pinguis **(**Ppi**)**	(AC19)	Mediterranean	4.5 ± 1.1	11.0 ± 0.3	0.7 ± 0.1
Pavlova sp. **(**Pth**)**	(AC 251)	Indian Ocean	3.8 ± 0.8	20.9 ± 1.0	0.9 ± 0.1
Rebecca salina **(**Rsa**)**	(AC87)	North Atlantic	4.4 ± 1.0	12.6 ± 2.1	0.8 ± 0.1

2.2 Feeding trials with C. gigas and P. maximus larvae

Pacific oyster larvae were obtained from previously conditioned broodstock. Conditioning methods and fecundation procedures were similar as those described by Robert et al. (2001). Embryonic development was carried out at 24 °C in 150-l tanks containing gently aerated seawater (1 µm filtered, salinity 34-35‰) at a density of 50 larvae ml^{-1}. Feeding trials with *C. gigas* were carried out with larvae with an initial age of 2 days. Larvae were kept in 2-l glass beakers at a density of 5 ml^{-1} and maintained at 24°C. During feeding trials, each lasting 14 days, pavlovophytes were tested in mixed diets, each running in triplicate, in association with the diatom *C. calcitrans* forma *pumilum*. Indeed, single diets does not allow sufficient *C. gigas* larval growth and most often high mortality occurs in such monospecific feeding experiment, leading to bias resulting in data difficulty interpretation. *C. calcitrans forma pumilum* is a very high nutritional diatom for *C. gigas* (Robert et al., 2005) and, when used in mixtures, cautions must be taken to do not mask the effect of the other microalgae. To point out the food value of the different pavlophytes without diatom overlap the bispecific diets were accordingly composed of 80% flagellate and 20% diatom; based on cellular volume, all microalgae were similar when used. This method was preferred to that of dry weight equivalent because rapid checking could be performed easily on all phytoplanctonic cultures using a Coulter counter linked to a channelyser. *C. gigas* larvae were fed with microalgae harvested in exponential phase. Different control groups were included in each trial: starved larvae as a negative control, and a group fed a standard diet (*C. calcitrans* f. *pumilum* + *Isochrysis* aff. *galbana* clone T-Iso) as a positive control (Robert et al., 2005). In addition, the diatom was included as the sole component in two other control groups, one fed 20% (corresponding to the diatom quantity included in bispecific diets) and one at 100% ration. In such small volume it is not recommended to aerate the culture while an excess of microalgae has negative effect on larval development (Helm and Bourne, 2004). In 2-l beakers no air was accordingly supplied while food was delivered every other day, at each seawater renewal; the standard daily food ration gave a total cell concentration of 50 cells $µl^{-1}$ for the first week and 100 cells $µl^{-1}$ for the second week which is sufficient to cover the demand at a density of 5 $larvae.ml^{-1}$.

An additional feeding trial was set up in order to verify the potential toxicity of *P. pinguis* and *R. salina* exudates for *C. gigas* larvae. Cultures of both strains were harvested during the exponential phase of growth and then centrifuged (3200 *g*, 5 min); thereafter, 20 ml of the

supernatant were added to larvae together with a monospecific diet of *C. calcitrans* f. *pumilum*, while the pellets were resuspended in 1-µm filtered seawater and used to feed another control group.
Larvae were sampled at the start, after one week and at the end of the feeding trials. At each sampling time at least 200 larvae were collected for each replicate in order to assess mortality by counting the translucent larval shells, and to measure the major length, defined as the antero-posterior axis parallel to the hinge, by means of image analysis. For this purpose a digital camera was connected to a microscope and acquired images were analysed with the IMAQ Vision Builder software (National Instruments).
Lastly, to check the effective ingestion of the different pavlophytes, a grazing test was also performed on *C. gigas* larvae. This sole trial was set up to verify if the poor food value recorded in the previous trials could be related to microalgae uptake. A batch of larvae were grown in 150-l tanks and fed with a multispecific diet composed of T-Iso and *C. calcitrans* f. *pumilum*. After a day of starvation, larvae aged 16 days were transferred, at a density of 5 ml^{-1}, into 30-l cylindro-conic tanks, each containing a pavlovophyte at an initial density of 100 cell μl^{-1}. In such volume constant air-bubbling was supplied to avoid sedimentation of microalgal cells. A sample from each tank was collected hourly, sieved (20 µm) to remove larvae, and total particles counted with a Counter coulter (ZN) during an 8h period.
P. maximus larvae were obtained from a commercial hatchery (Tinduff, France) and were 4 days old at the beginning of the feeding trials. Rearing and analysis methods were similar as those described for *C. gigas* larvae, except for a lower temperature (18 °C) and the addition each 2 days of thiamphenicol (7 mg l^{-1}) to limit bacterial contamination. Moreover, the diets based solely on the diatom were excluded from the trial.

2.3 Analytical procedures

Phytotechnical and biochemical analyses were performed in duplicate with biomass harvested after six days of culture at which point cultures were in exponential or late exponential phase of growth. Microalgae biochemical composition was examined concurrently with larval feeding experiments which means that for each species three samples were performed during larval development. Growth was estimated daily using a Malassez haemocytometer; growth rate was calculated as: $\mu = [(\ln N_1 - \ln N_0)/(t_1 - t_0)]/\ln 2$, where N_1 = measurement at time 1 (t_1), N_0 = measurement at time 0 (t_0). Cellular size, expressed in diameter equivalent, was determined using a Channelyser C256.
Dry weight measurements were obtained by centrifuging (3200g, 10 min) 50 ml of culture; pellets were then rinsed with 20 ml of ammonium formate 0.5 M, centrifuged again and then transferred into pre-weighed tin capsules. Dry weight and ash content were measured after heating at 80°C (overnight) and 450 °C (4 h), respectively. For gross composition, 50 ml of culture were harvested by centrifugation (3000 r.p.m., 10 min); pellets were stored at -20 °C for a period of up to six months prior to analysis. Proteins, lipids and carbohydrates were analysed using the methods described by Lowry et al., (1951), Dubois et al. (1956) and Bligh and Dyer, (1959), respectively. For fatty acid and sterol analyses, samples of 10-50 ml of culture, according to cellular concentration, were filtered through 47-mm precombusted (450 °C for 8 h) Whatman GF/F glass-fibre filters and stored at -20 °C in chloroform/methanol (2/1) for a period of up to six months prior to analysis. For fatty acid analysis, neutral and polar lipids were separated in a silica gel microcolumn (30 mm x 5 mm; Kieselgel Merck, 70-230 µm mesh), previously heated to 450 °C and deactivated with 5% water, as described by Soudant *et al.* (1995). Fatty acid analyses were performed according to the method described by Marty et al. (1992), using a Hewlett-Packard HP6890 gas-chromatograph, equipped with a J&W 65 DB Wax column (30 m x 0.25 mm; 0.25 µm film thickness), using hydrogen as the

carrier gas and 23:0 as the internal standard. Sterols of the neutral lipid fraction were analysed according to Soudant et al. (2000), using a Chrompack CP 9002 gas chromatograph equipped with a Restek Rt X 65 fused silica capillary column (15 m x 0.25 mm, 0.25 µm film thickness), using hydrogen as the carrier gas and cholestane as the internal standard.
Biochemical analysis results (gross, fatty acids and sterol compositions) were expressed in relative terms (percentage of organic matter or percentage of total fatty acids or total sterols) allowing comparisons independently of microalgae cells weight which increased from exponential to stationary phases. Total fatty acids and total sterols weights were also reported to allow quantitative terms calculations (weight of each component per cell = total weight* relative composition).

2.4. Statistics

Significant differences between feeding experimental conditions were detected by means of ANOVA and Scheffe tests ($P<0.05$) and data expressed in percentage previously transformed (arcsine square root x_i 100^{-1}) before statistical treatments.

3. Results

3.1 Size, growth, biochemical composition of the microalgae

Cellular size of the pavlovophyte strains used varied between 3.8 (Pth) and 5.3 µm (Pfl) in equivalent diameter, while dry weight ranged from 14.8 (Pfl) to 28.8 pg cell^{-1} (Psh) (Tables 1 and 2). After six days of culture Pth exhibited the highest growth rate (0.9 division day^{-1}), reaching a concentration of 20.9 x10^6 cell ml^{-1}. The other species exhibited a similar growth rate (0.7-0.8 division day^{-1}) and reached concentrations of 9.7-13.4 x10^6 cell ml^{-1}.
Protein content of the six strains ranged from 33.8-54.3% of organic matter, and thus represented the main chemical component, except for Pth, where carbohydrates were dominant. Carbohydrates accounted for 30.2-44.2% of organic matter, while lipids showed the most variability between species, ranging between 15.5% (Pfl) and 29.2% (Psh) of organic matter content.

Table 2. Dry weight, ash content and gross composition of the six pavlovophytes used in the present feeding trials (mean ± S.D.; n ≥ 2).

	Pfl	**Psh**	**Psm**	**Ppi**	**Pth**	**Rsa**
Dry weight (pg cell^{-1})	14.8 ± 0.4	28.8 ± 0.5	20.5 ± 1.4	23.0 ±0.8	20.2 ± 0.4	18.2 ± 1.1
Ash (% dry weight)	6.9 ± 0.8	13.5 ± 0.8	13.9 ± 0.5	5.2 ± 0.2	5.8 ± 0.4	5.0 ± 0.2
Proteins (% organic matter)	54.3 ± 2.5	41.3 ± 0.7	40.2 ± 6.6	38. 1± 1.4	33.8 ± 4.8	44.6 ± 0.1
Carbohydrates (% o.m.)	30.2 ± 0.7	29.5 ± 1.1	36.3 ± 3.5	38.2 ± 1.2	44.2 ± 3.3	32.3 ± 0.3
Lipids (% o.m.)	15.5 ± 1.8	29.2 ± 1.8	23.5 ± 3.1	23.8 ± 0.2	22.0 ± 1.5	23.1 ± 0.2

The six microalgae exhibited differences in fatty acid contents, ranging from 6.4% (*R. salina*) to 16.3% (Pth) of organic matter (Table 3). In contrast, fatty acid composition was broadly similar among the different strains, with some exceptions. The main fatty acids found were the saturated 14:0, 16:0, the monounsaturated 16:1(n-7) and the polyunsaturated EPA; these fatty acids accounted for more than 60% of total content. The polyunsaturated fraction formed the main fatty acid component in all species (35-56% of total), while the saturated and monounsaturated fractions accounted for 28-39% and 13-31% of total fatty acids, respectively. As concerns PUFA, all species were rich in EPA (≥ 15% of total), while DHA varied significantly among different strains; in particular Psh and Pfl exhibited high DHA content (≈ 9-10% of total fatty acids), while in Psm and in *P. pinguis* this fatty acid accounted for only 3-4%. The PUFA contents of the six strains expressed as a percentage of total organic matter content are illustrated in Fig.1. The highest PUFA content was found in Pth, followed by Pfl, Psm, *P. pinguis*, *R. salina* and Psh, in that order.

Neutral sterols of the six strains represented 0.4-2.1% of organic matter content (Table 4). Among sterols, stigmasterol was found to be dominant in all species (≥ 53% of total), while both methylporiferasterol and β-sitosterol were well represented, with values of 8-15% and 5-12%, respectively. On the other hand, campesterol was found in small quantities (2-3% of total) in all strains, while other sterols varied greatly among different strains. Pfl and Psh were particularly rich in 24-methylenecholesterol, Psm, *P. pinguis* and *R. salina* contained high quantities of ethylpavlovol, while Pth exhibited a significant content of β-sitosterol.

Fig. 1. PUFA content of the six pavlovophytes used in the present trials. Data are expressed as percentage of organic matter content (n = 2).

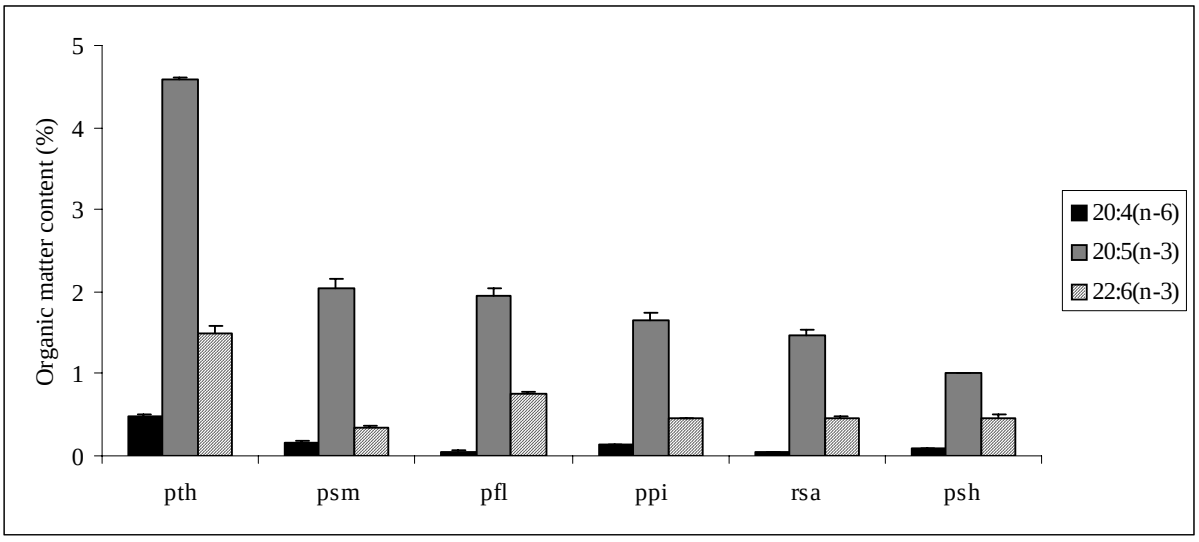
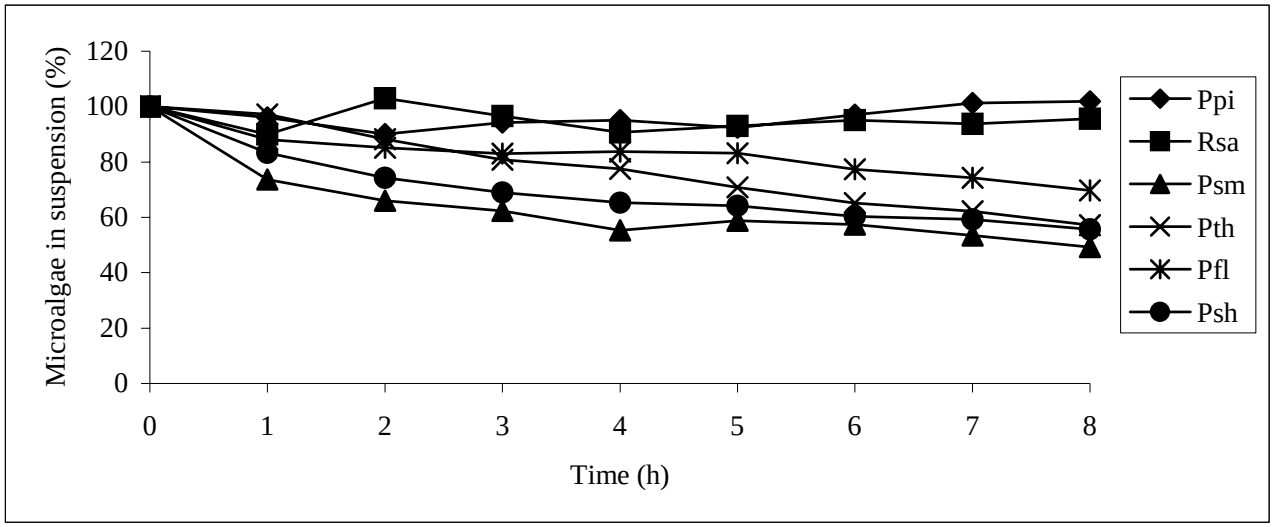


Fig. 2. Clearance of *Crassostrea gigas* larvae fed six different pavlovophytes over an 8 h period. Pfl = *Pavlova sp.* AC 250, Psh = *Pavlova sp.* AC248, Pth = *Pavlova sp.* AC 87, Psm = *Pavlova sp.* AC 538, Ppi = *Pavlova pinguis* and Rsa = *Rebecca salina* (*ex Pavlova salina*).



Both of the pavlovophyte strains tested during the first trial did not exhibit any nutritional value for *C. gigas* larvae (Table 5), similar to unfed larvae and by far lower then larvae fed Cp20 diet. The possibility that exudates from cultures of *P. pinguis* or *R. salina* were toxic was excluded by the second feeding trial results (Table 6). Both diets including the supernatant of these microalgae combined with *C. calcitrans* f. *pumilum* gave high survival and growth comparable to that obtained with the diatom only. In contrast, larvae fed with *R. salina* or *P. pinguis* cells centrifuged and resuspended in fresh medium exhibited poor growth during the first week and even higher mortality during the second week. The new species included in this trial (Psh) exhibited also a poor nutritional value for *C. gigas* larvae, with low growth compared to Cp20 diet and low survival after two weeks of rearing.

Table 3. Fatty acid composition (mean ± SD; n = 2) of the six pavlovophytes used in the present trials Individual data are reported as percentage of total fatty acids while total fatty acids (TO FA) are expressed either total dry weight or percentage of total organic matter content (o.m.).

	Pfl	**Psh**	**Psm**	**Ppi**	**Pth**	**Rsa**
14:0	17.8 ± 0.3	10.7 ±0.2	17.8 ± 0.5	15.9 ± 0.6	12.1 ± 1.0	20.0 ± 0.6
16:0	12.9 ± 0.8	16.5 ±0.1	14.5 ± 1.2	17.3 ± 0.8	26.5 ± 0.9	11.8 ± 0.4
16:1(n-7)	9.0 ± 0.5	11.3 ±0.4	28.2 ± 0.9	22.9 ± 0.7	15.2 ± 0.4	19.5 ± 0.3
16:2(n-4)	1.4 ± 0.3	1.4 ±0.1	1.5 ± 0.2	1.1 ± 0.1	0.9 ± 0.1	2.1 ± 0.1
18:2(n-6)	1.1 ± 0.2	2.4 ±0.1	1.8 ± 0.2	2.5 ± 0.1	3.6 ± 0.1	1.2 ± 0.1
18:3(n-3)	1.3 ± 0.2	0.4 ±0.1	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
18:4(n-3)	10.1 ± 0.7	6.1 ±0.1	4.0 ± 0.7	4.5 ± 0.1	3.6 ± 0.1	4.9 ± 0.5
20:4(n-6)	0.6 ± 0.1	2.2 ±0.1	1.3 ± 0.1	1.2 ± 0.1	2.1 ± 0.1	0.6 ± 0.1
20:5(n-3)	22.6 ± 1.2	22.5 ±0.2	15.4 ± 1.0	15.6 ± 0.9	19.6 ± 0.1	19.5 ± 0.8
22:5(n-6)	8.3 ± 0.4	7.8 ±0.7	5.3 ± 0.1	7.2 ± 0.2	4.9 ± 0.1	6.8 ± 0.1
22:6(n-3)	8.8 ± 0.3	10.2 ±1.1	2.6 ± 0.1	4.3 ± 0.1	6.4 ± 0.4	6.2 ± 0.1
TO.SAT.	31.8 ± 1.5	27.6 ± 0.4	33.18 ± 1.61	33.9 ± 2.1	39.0 ± 0.3	32.8 ± 1.3
TO.MONO	12.5 ± 0.9	16.7 ± 0.8	31.24 ± 1.18	26.1 ± 1.2	17.8 ± 0.1	22.1 ± 0.1
TO.(n-9)	0.8 ± 0.1	0.9 ± 0.1	1.1 ± 0.2	1.9 ± 0.2	1.2 ± 0.1	1.3 ± 0.1
TO.(n-7)	9.3 ± 1.0	11.5 ± 0.4	28.6 ± 0.9	23.3 ± 1.0	15.2 ± 0.5	19.8 ± 0.3
TO.POLY	55.5 ± 1.3	55.7 ± 1.2	35.4 ± 2.7	40.1 ± 1.7	42.9 ± 0.1	45.0 ± 1.2
TO.(n-4)	1.6 ± 0.2	1.5 ± 0.1	1.9 ± 0.4	1.2 ± 0.1	1.0 ± 0.1	2.3 ± 0.1
TO.(n-6)	10.4 ± 0.6	14.6 ± 0.2	10.4 ± 0.2	13.2 ± 0.8	11.6 ± 0.2	11.0 ± 0.2
TO.(n-3)	43.3 ± 1.5	39.6 ± 1.1	22.9 ± 2.1	25.6 ± 1.4	30.0 ± 0.3	31.5 ± 1.4
(n-3)/(n-6)	4.2 ± 0.1	2.7 ± 0.4	2.2 ± 0.2	1.9 ± 0.1	2.6 ± 0.1	2.9 ± 0.2
22:6/20:5	0.4 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
22:5/20:4	13.6 ± 0.3	3.6 ± 0.45	4.00 ± 0.2	5.8 ± 0.2	2.3 ± 0.3	12.4 ± 0.4
TO FA weight (fg cell^{-1})	1140.0 ± 42.1	1109.9 ± 76.8	2333.2 ± 286.5	2303.4 ± 134.9	4303.0 ± 235.6	1311.4 ± 16.2
TO FA (% o.m)	7.7 ± 0.6	15.9 ± 1.0	10.7 ± 2.3	10.0 ± 1.1	16.3 ± 0.9	6.4 ± 0.3

3.2 Feeding trials

Table 4. Sterol composition of the six pavlovophytes used in the present feeding trials (mean ± SD; n=2). Individual data are reported as percentage of total sterols while total sterols (TO sterols) are expressed either total dry weight (fg cell^{-1}) or percentage of total organic matter content (% o.m.).

	Pfl	**Psh**	**Psm**	**Ppi**	**Pth**	**Rsa**
CHOL	2.9 ± 0.4	5.4 ± 0.3			1.0 ± 0.1	
BRA		0.5 ± 0.1			1.4 ± 0.1	
DES		0.4 ± 0.1			2.0 ± 0.1	
CAM	2.3 ± 0.4	3.9 ± 0.1	2.8 ± 0.1	2.1 ± 0.1	2.6 ± 0.1	1.8 ± 0.1
24-MET	19.3 ± 0.7	12.5 ± 0.1		5.9 ± 0.5		
STI	53.1 ± 0.3	52.8 ± 0.1	59.2 ± 0.2	59.5 ± 0.4	60.1 ± 0.2	64.1 ± 2.4
MeP	15.1 ± 0.5	10.5 ± 0.1	10.9 ± 0.1	8.3 ± 0.1	16.4 ± 0.3	11.0 ± 0.7
β–SI	5.9 ± 0.9	11.6 ± 0.1	9.9 ± 0.5	10.2 ± 0.4	4.9 ± 0.1	7.8 ± 0.3
FUC		1.1 ± 0.1	4.5 ± 0.1	3.8 ± 0.2	10.1 ± 0.1	4.2 ± 0.3
ISOFUC		0.9 ± 0.2	0.5 ± 0.1	0.4 ± 0.1		
MET			1.4 ± 0.1	1.2 ± 0.1	0.7 ± 0.1	1.5 ± 0.2
ETH	1.5 ± 0.1	0.5 ± 0.1	10.8 ± 0.2	8.5 ± 0.1	1.0 ± 0.1	9.6 ± 1.3
TO sterol weight (fg cell^{-1})	192.1 ± 37.8	444.3 ± 0.1	336.9 ± 47.8	352.8 ± 26.8	394.3 ± 24.5	298.0 ± 3.0
TO sterol (% o.m.)	1.5 ± 0.3	1.8 ± 0.1	1.9 ± 0.3	0.4 ± 0.1	2.1 ± 0.1	1.7 ± 0.1

CHOL = Cholesterol (Cholesta-5-en-3β-ol);
BRA = Brassicasterol (24β-methylcholesta-5,22-dien-3β-ol);
DES = Desmosterol (cholesta-5-en-3β-ol);
CAM = Campesterol (24α-methylcholesta-5-en-3β-ol);
24-MET = 24-Methylenencholesterol (24β-methylcholesta-5,24(28)-dien-3β-ol);
STI = Stigmasterol (24β-ethylcholesta-5,22-dien-3β-ol);
MEP = 4α–Methylporiferastenol (4α-methyl-24α-ethylcholesta-22-en-3β-ol);
β-SI= β–Sitosterol (24β-ethylcholesta-5-en-3β-ol);
FUC = Fucosterol (24β-ethylcholesta-5,24(28)-dien-3β-ol);
ISOFUC= Isofucosterol (24β-ethylcholesta-5,24(28)-dien-3β-ol);
MET = methylpavlovol (4α,24β-dimethylcholestan-3β,4β-diol);
ETH = ethylpavlovol (4α-methyl-24β-ethylcholestan-3β,4β-diol).

Table 5 Length and survival (average ± S.D.) of *C. gigas* larvae fed different diets. Initial shell length = 83.0 ± 5.5 µm. Values within the same column sharing a common superscript letter are not significantly different (*P*>0.05). **Cp+Ti**: *C. calcitrans* f. *pumilum* + T-Iso; **Cp+Rsa**: *C. calcitrans* f. *pumilum* + *R. salina;* **Cp+Ppi***: C. calcitrans* f. *pumilum* + *P. pinguis;* **Cp100** *C. calcitrans* f. *pumilum* (100% of ration); **Cp20** *C. calcitrans* f. *pumilum* (20% of ration); **Unfed**: starved larvae**.**

	First week		Second week	
	length	survival	length	survival
cp+Ti	125.8 ± 20.1^{a}	91.1 ± 0.9^{b}	175.1 ± 42.1	89.7 ± 0.5^{a}
cp+Rsa	87.3 ± 5.3^{b}	84.0 ± 7.4^{c}	89.0 ± 5.2^{a}	20.1 ± 2.8
cp+Ppi	85.1 ± 4.9^{b}	81.7 ± 5.0^{c}	87.1 ± 3.9^{a}	8.3 ± 1.6
cp100	129.3 ± 19.5^{a}	94.2 ± 1.2^{ab}	149.5 ± 29.8	89.1 ± 0.6^{ab}
cp20	110.9 ± 12.3	93.2 ± 1.9^{ab}	121.3 ± 15.0	83.1 ± 1.4^{b}
Unfed	91.6 ± 6.8	95.7 ± 1.3^{a}	92.1 ± 6.7^{a}	84.3 ± 3.0^{ab}

Table 6 Length and survival (average ± S.D.) of *C. gigas* larvae fed different diets. Initial shell length = 81.1 ± 4.4 µm. Values within the same column sharing a common superscript letter are not significantly different (*P*>0.05). **Cp+Ti**: *C. calcitrans* f. *pumilum* + T-Iso; **Cp+Pps**: *C. calcitrans* f. *pumilum* + *P. pinguis* surnatant*;* **Cp+Rss***: C. calcitrans* f. *pumilum* + *R. salina* surnatant*;* **Cp+Ppc**: *C. calcitrans* f. *pumilum* + *P. pinguis* resuspended*;* **Cp+Rss***: C. calcitrans* f. *pumilum* + *R. salina* resuspended*;***Cp20** *C. calcitrans* f. *pumilum* (20% of ration); **Cp+Pps**: *C. calcitrans* f. *pumilum* + *Pavlova sp.* AC248; **Unfed**: starved larvae.

	First week		Second week	
	length	survival	Length	survival
Cp+Ti	127.0 ± 14.0	98.5 ± 0.3^{ab}	160.7 ± 14.0	95.9 ± 1.2^{a}
Cp+Pps	115.7 ± 11.3^{ab}	98.8 ± 0.4^{ab}	137.7 ± 17.0^{a}	95.4 ± 2.1^{a}
Cp+Rss	117.6 ± 11.3^{a}	98.9 ± 0.8^{ab}	139.5 ± 15.0^{a}	96.8 ± 1.5^{a}
Ppc	83.0 ± 3.9^{c}	92.2 ± 5.9^{b}	n.d.	6.8 ± 2.0^{b}
Rsc	83.0 ± 4.8^{c}	92.9 ± 4.7^{b}	n.d.	7.4 ± 2.1^{b}
Cp20	115.0 ± 11.2^{b}	96.3 ± 1.3^{ab}	135.9 ± 14.6^{a}	96.3 ± 1.0^{a}
Cp+Psh	98.7 ± 9.0	99.3 ± 0.4^{ab}	102.6 ± 10.4	23.3 ± 11.7
Unfed	87.4 ± 4.0	99.5 ± 0.5^{a}	88.2 ± 4.3^{d}	90.2 ± 5.9^{a}

After two weeks of rearing among the three species tested in the third feeding trial (Table 7) only Pfl gave growth and survival similar to the reference diet Cp only, while the others resulted in lower larval performances.

Grazing by *C. gigas* larvae varied for the six strains (Fig. 1). In particular, *P. pinguis* and *R. salina* were weakly ingested and more than 95% of cells still remained in suspension eight hours after delivery. In contrast, the four other strains were more efficiently ingested (30-50% of cells ingested eight hours latter).

Table 7 Length and survival (average ± S.D.) of *C. gigas* larvae fed different diets. Initial shell length= 78.2 ± 4.8 µm. Values within the same column sharing a common superscript letter are not significantly different (*P*>0.05). **Cp+Ti**: *C. calcitrans* f. *pumilum* + T-Iso; **Cp+Pfl**: *C. calcitrans* f. *pumilum* + *Pavlova sp.* AC 250; **Cp+Pth***: C. calcitrans* f. *pumilum + Pavlova sp.* AC 251*;* **Cp+Psm***: C. calcitrans* f. *pumilum + Pavlova sp.* AC;**Cp100** *C. calcitrans* f. *pumilum* (100% of ration); **Cp20** *C. calcitrans* f. *pumilum* (20% of ration); **Unfed**: starved larvae.

	First week		Second week	
	length	survival	length	survival
cp+Ti	112.3 ± 23.15^{a}	79.4 ± 4.7^{b}	192.8 ± 41.8	62.9 ± 4.5^{ab}
cp+Pfl	100.2 ± 16.41	86.2 ± 2.5^{ab}	140.5 ± 36.7^{a}	57.9 ± 11.8^{ab}
cp+Pth	92.0 ± 10.1^{b}	90.6 ± 4.9^{ab}	117.7 ± 24.5	57.7 ± 11.0^{ab}
cp+PSm	88.0 ± 9.7^{b}	90.2 ± 2.8^{ab}	103.3 ± 24.2	42.0 ± 6.3^{b}
cp100	117.1 ± 26.2^{a}	87.3 ± 6.4^{ab}	159.7 ± 35.7	64.4 ± 6.2^{ab}
cp20	113.1 ± 20.2^{a}	92.5 ± 2.6^{ab}	143.5 ± 23.1^{a}	71.2 ± 1.1^{a}
Unfed	81.4 ± 4.6	95.4 ± 3.4^{a}	83.9 ± 4.0	58.0 ± 2.6^{ab}

After one week of *P. maximus* larvae rearing, the low food value of *R. salina, P. pinguis*, Psm and Pth was confirmed, resulting in growth similar to unfed larvae (Table 8). Larvae fed Cp+Psh diet exhibited similar growth to positive control (Cp+Ti), while larvae fed Cp+Pfl showed better growth rates. During the second week, the best larval performances on Cp+Pfl diet were confirmed. No significant differences were found among the control diet Cp+Ti and Cp+Psh, Cp+Pth and Cp+Rsa, while larvae fed *P. pinguis* or Psm exhibited development similar to unfed larvae. However, these latter strains exhibited unusual growth during the trial, possibly due to the occurrence of an unusual picoplancton bloom in the hatchery reservoir.
The use of antibiotics during the trial resulted in high survival (≥ 91%) in all cases.

Table 8 Length and survival (average ± S.D.) of *P. maximus* larvae fed different diets. Initial shell length= 93.7 ± 10.2 µm. Values within the same column sharing a common superscript letter are not significantly different (*P*>0.05).

	First week		Second week	
	length	survival	length	survival
Cp+Ti	133.7 ± 20.1^{a}	96.1 ± 1.3^{a}	164.0 ± 34.4^{a}	88.6 ± 1.1^{a}
cp+Pfl	142.8 ± 29.8	98.0 ± 2.1^{a}	208.0 ± 38.4	95.1 ± 0.5^{a}
cp+Ppi	122.5 ± 16.6^{b}	96.7 ± 0.8^{a}	140.4 ± 26.4^{c}	92.3 ± 1.4^{a}
cp+Psh	134.6 ± 20.4^{a}	97.7 ± 0.6^{a}	164.0 ± 37.5^{a}	93.4 ± 2.7^{a}
cp+Psm	122.1 ± 16.2^{b}	96.9 ± 0.3^{a}	146.9 ± 21.0^{bc}	92.2 ± 3.8^{a}
cp+Pth	124.4 ± 17.0^{b}	96.6 ± 0.6^{a}	158.1 ± 31.3^{ab}	93.1 ± 2.2^{a}
cp+Rsa	121.0 ± 18.5^{b}	98.5 ± 0.5^{a}	152.8 ± 24.1^{ab}	91.9 ± 0.5^{a}
Unfed	120.5 ± 15.7^{b}	96.6 ± 0.6^{a}	139.5 ± 23.5^{c}	90.6 ± 1.2^{a}

3. Discussion

At present, a certain level of mastery of the main procedures employed in mollusc hatcheries has been reached (Helm and Bourne, 2004), but nevertheless several constraints remain unsolved. In particular, larval rearing is strictly dependent on phytoplankton production, an activity that requires considerable human and economic investment. The number of microalgal strains routinely used in hatcheries is extremely limited, particularly as concerns flagellates, and the search for suitable new strains is clearly a priority. The effectiveness of microalgae as food for mollusc larvae depends on several criteria such as size, shape, availability in the water column, digestibility, biochemical profile and culture productivity (Webb and Chu, 1983; Brown et al., 1989, Robert et al., 2004). The haptophyte class Pavlovophyceae includes species that potentially fulfil these criteria.

The nutritional value for molluscs of the pavlovophytes *P. lutheri*, *P. pinguis* and *R. salina* has been widely investigated (Walne, 1970; Langdon and Waldock, 1981; Brown et al., 1998; Jonsson et al., 1999; McCausland et al., 1999; Heasman et al., 2000; Ponis et al., 2003a,b), and more recently another pavlovophyte, *Diacronema vlkianum*, has been shown to be of interest for *C. gigas* larvae (Ponis et al., 2005). In the present study, various characteristics (size, cell density, productivity, biochemical composition) of six pavlovophytes have been assessed and their food value for *C. gigas* and *P. maximus* larvae examined.

All strains included in this study were of appropriate size (3.8-5.3 µm) to be ingested by *C. gigas* larvae and exhibited high productivities, reaching at least a concentration of 10 x 10^6 cells ml^{-1} after six days of culture in 2-l carboys. Similar values have been reported for the flagellate strain T-Iso, which is routinely cultured in mollusc hatcheries and considered as a reference strain.

All of the tested microalgae exhibited high protein and carbohydrate contents (34-54% and 30-44% of organic matter, respectively). These levels are similar to those reported in the literature for bivalve requirements. For example, Brown et al. (1989) stated that microalgal protein content should account for 30-60% of dry weight, while Utting (1986) noted that high carbohydrate contents are required for acceptable larval *C. gigas* development. The gross composition of *P. pinguis* measured here was coherent with the values recorded for the same species by McCausland et al. (1999), while *R. salina* lipid content was similar to that analysed by Volkman et al. (1991). The six pavlovophytes studied here showed similar fatty acid profiles. All species were rich in 14:0, 16:0 and EPA (48-58% of total fatty acids), while DHA content varied significantly among species; these fatty acids are fundamental for oyster normal larval and post-larval developments (Langdon and Waldock, 1981, Thompson et al., 1993). The fatty acid composition of *P. pinguis* measured in this study was similar to that reported by Volkman et al. (1997), while higher saturated and monounsaturated contents and lower polyunsaturated fatty acid levels (with particular reference to EPA and DHA) were reported for the same species by McCausland et al. (1999). Apart from a lower content of 16:1(n-7) and higher concentrations of 18:4(n-3) EPA and DHA reported here, the fatty acid profile of *R. salina* corresponds to that reported for the same species by Volkman et al. (1991). Ethylpavlovol was detected in all six pavlovphytes, while methylpavlovol was found in only four strains (Psm, *P. pinguis*, Pth, *R. salina*). These sterols, specific to members of the order *Pavlovales*, are however poorly incorporated by *C. gigas* spat or *Pecten maximus* larvae (Soudant et al., 1998; Knauer et al., 1999) and this fact might partially explain their low nutritional value. The sterol profile of *P. pinguis* measured in our study was similar to that

described by Volkman et al. (1997), but different from that reported by Ghosh et al. (1998) for the same species.
In spite of balanced biochemical profiles, bispecific diets including the different pavlovophytes were of poor food value for *C. gigas* and in fact, except for the diet including Pfl, the nutritional value of the associated diatom was depressed (i.e. lower larval performances than that obtained with the control Cp20 only). *P. pinguis* and *R. salina* were poorly ingested, as shown in eight hour larval feeding trials. No negative effects of exudates of cultures were detected, and thus the low ingestion rate was apparently not due to exo-toxin production. Similar observations on *C. gigas* larvae were reported for *P. lutheri*: no negative effects of exudates (Ponis et al., 2003b) but low cells consumption (Robert et al., 2005). During feeding trials with *Pecten maximus* larvae, *P. pinguis* and Psh led to poor larval development, similar to that of unfed larvae. After two weeks of rearing, unfed larvae exhibited a significant increase in shell length. An abnormal picoplancton bloom was detected in the hatchery reservoir and despite fine water filtration (sequential cartridge filtration at 5 and 1 µm) an increase of organic particulates and/or natural phytoplankton in filtered seawater was suspected. *Pecten maximus* larvae fed with Pfl exhibited relatively high survival and significantly greater growth after two weeks of rearing than that measured with the reference diet Cp+Ti.
Although several studies on the nutritional value of microalgae and basic nutritional requirements of reared molluscs have been carried out, their relationships remain poorly defined. The food value of a given microalga depends on both the mollusc species and growth stage considered (Brown et al., 1997; Knauer and Southgate, 1999; Muller-Feuga et al., 2003). For example, in the present study the low food value of *R. salina* for *C. gigas* and *P. maximus* larvae has been demonstrated. However, this species has been reported to be of interest when used as food for *Pecten margaritifera* larvae and for *C. gigas* juveniles (Brown et al., 1998; Doroudi et al., 2003).
This study clearly indicates that cultures of members of the class *Pavlovophyceae* are unsuitable for larval *C. gigas* feeding, leading to poor growth and low survival. In contrast, promising results for *P. maximus* larval rearing were obtained with a new pavlovophyte strain. Further investigations on its productivity in commercial operating conditions should be performed in order to promote its use in hatcheries.

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