

Original article

**Fermentation of green alga sea-lettuce (*Ulva* sp) and metabolism of its sulphate by human colonic microbiota in a semi-continuous culture system**

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**Summary** — The green alga, sea-lettuce (*Ulva* sp), could be considered as a new source of dietary fibre. *Ulva*, however, contains high levels of sulphate, part of which is chemically bound in soluble polymers (ulvan). The purpose of this study was to assess the fermentation characteristics and sulphate metabolism of *Ulva* and ulvan by human faecal bacteria fermentation system using a semi-continuous fermenter. *Ulva* and ulvan were poorly fermented, even after adaptation of the microbiota. Only 16.6% and 8.9% of *Ulva* and ulvan organic matter, respectively, were recovered as short chain fatty acids. Nevertheless, 40% of the sulphate in *Ulva* was dissimilated to sulphide by sulphate-reducing bacteria. Supplementation of *Ulva* with more fermentable polysaccharides, such as algal xylan and resistant starch, though decreasing the ammonia production originating from *Ulva* protein degradation, did not significantly reduce the sulphide levels. It is postulated that unless crude *Ulva* is desulphated, its daily consumption at a level of 20 g of dry product could stimulate colonic microbial sulphate reduction, which may have detrimental effects for the host.

**in vitro fermentation / *Ulva* / seaweed / colonic bacteria / sulphate reduction**

**Résumé** — Étude en culture semi-continue de la fermentation de l'algue verte, laitue de mer (*Ulva* sp), et du métabolisme de ses sulfates par les microorganismes du côlon humain. L'algue verte, laitue de mer (*Ulva* sp), peut être considérée comme une nouvelle source de fibre alimentaire. Cependant, *Ulva* contient une teneur élevée en sulfates dont une partie est liée chimiquement à un ensemble de polymères solubles (ulvane). L'objet de cette étude a été d'étudier les caractéristiques fermentaires

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et le métabolisme du sulfate d'*Ulva* et d'ulvane en utilisant un système de fermentation semi-continu. *Ulva* et ulvane se sont montrés peu fermentescibles, même après adaptation de la microflore. Seuls 16,6 % de la matière organique d'*Ulva* et 8,9 % de celle d'ulvane ont été retrouvés sous forme d'acides gras à chaîne courte. Néanmoins, 40 % des sulfates contenus dans *Ulva* ont été transformés en sulfure par les bactéries sulfatoréductrices. La supplémentation d'*Ulva* par des glucides plus fermentescibles tels que du xylane d'algue et de l'amidon résistant, bien que diminuant la production d'ammoniac due à la dégradation des protéines d'*Ulva*, n'a pas limité significativement la formation de sulfure. En conclusion, la consommation journalière de 20 g d'*Ulva* brute sèche, pourrait accroître la sulfatoréduction par la flore colique, ce qui pourrait avoir des conséquences défavorables pour l'hôte, à moins que ce produit ne soit préalablement désulfaté.

### **fermentation in vitro / *Ulva* / algue / flore colique / sulfatoréduction**

## **INTRODUCTION**

There is considerable evidence to support the view that fermentation of dietary fibres by anaerobic bacteria in the human colon contributes to the prevention of colonic diseases. Short chain fatty acids (SCFA), which are the principal end products of fermentation, have a variety of recognized physiological and clinical properties (Cummings, 1995). However, wide ranges of fermentability exist among different types of dietary fibres, and the action of an individual fibre depends on the extent of its breakdown, as well as on the pattern of its fermentation end products. For example, beneficial effects such as increase in stool output, dilution of colonic contents and production of distal colonic SCFAs are mainly associated with a low extent, or a slow rate of fermentation (Edwards, 1995). In this respect, the green alga, sea-lettuce (*Ulva* sp), could be considered as a new interesting source of dietary fibre. This organism contains about 30% total dietary fibre of which 16.5% are soluble polysaccharides (Lahaye, 1991). Recent investigation has shown that *Ulva* and its soluble fibres, referred to as ulvan, were poorly fermented in vitro in batch culture with faecal bacteria (Bobin-Dubigeon et al, 1996). Their particular chemical structures were suggested to explain this resistance to fermentation. How-

ever, such short-term studies do not take into account the potential capacity of intestinal bacterial communities to adapt to break down the polysaccharides (Edwards, 1995). It has been shown in rat experiments that short (1 week) or longer-term adaptations (up to 12 weeks) were necessary to achieve the greatest digestibility or rate of fermentation of specific fibres such as gums (Walter et al, 1986; Tulung et al, 1987; Brunsgaard et al, 1995) or fibres from canned peas (Monsma and Marlett, 1996). In humans, when gum arabic was included in the diet for 18 days, the proportion of the faecal flora able to degrade this substrate progressively rose from 6.5 to more than 50%. After the fibre was withdrawn from the diet, the proportion of gum arabic fermenting bacteria returned to the level present before ingestion (Wyatt et al, 1986). More recently, the necessity of adaptation of human subjects to xanthan gum, for their faecal microflora to achieve maximum hydrogen and SCFA production, was also demonstrated (Daly et al, 1993). Therefore, longer-term studies appear necessary to investigate whether adaptation of the flora occurs with this type of seaweed.

Another concern regarding *Ulva* is its high sulphate content, which can reach 17% of its dry matter (Bobin-Dubigeon et al, 1996). Part of this sulphate is chemically bound as in ulvan, which is a sulphated glu-

curonorhamnoglycan, constituting between 12 and 15% of the algal dry matter (Michel and Macfarlane, 1996), and containing about 14% sulphate (Ray and Lahaye, 1995). This attribute could have significant consequences on microbial metabolism in the colon and potential health implications. The supply of free sulphate to the colon resulting either from a high sulphate intake, eg, > 5 mmol/d (Florin et al, 1991) or from the in situ microbial degradation of sulphated polymers (Gibson et al, 1988a, 1991) may stimulate the growth and activity of sulphate-reducing bacteria (SRB) (Christl et al, 1992). These organisms reduce sulphate to sulphide. When sulphate is abundant, this reaction can represent an important route of hydrogen ( $H_2$ ) utilization in situ in the colon and may interact with other microbial pathways of  $H_2$  disposal, namely methanogenesis and reductive acetogenesis (acetate formation from  $CO_2$  reduction by  $H_2$ ) (Durand et al, 1995). In addition, hydrogen sulphide is toxic towards mammalian cells and it has been suggested that sulphide is a possible cofactor in the initiation and/or maintenance of the inflammatory bowel disease, ulcerative colitis (Gibson et al, 1991; Roediger et al, 1993). Consequently, knowledge of the fate of the sulphate associated with sea-lettuce is important to predict the potential health implications following its long-term consumption.

The purpose of the present study was to assess the effect of adaptation on the fermentation characteristics of *Ulva* and ulvan by human faecal bacteria, using a semi-continuous fermentation system. Emphasis was placed on the effect of these products on sulphate metabolism, and in order to ascertain more precisely the activities of SRB, the putatively specific inhibitor of sulphate reduction, sodium molybdate, was used. We also attempted to clarify if previous adaptation of the microflora to *Ulva* would affect its reductive acetogenic and methanogenic activities.

## MATERIALS AND METHODS

### Materials

*Ulva* sp, produced by Algues de Bretagne (France) as Laitue de mer was ground to pass through a 3 mm screen. It contained 90.4% dry matter (DM) and (% DM): minerals, 19.7; total nitrogen, 4.9; sulphate, 11.6. Crude ulvan was provided by CEVA (Pleubian, France) and further processed by precipitation by Inra (Nantes, France). It contained (% DM): minerals, 20.8; sulphate, 15.8. The red seaweed *Palmaria palmata* was also processed by CEVA in order to obtain a carbohydrate enriched product (70% DM), mostly composed of xylan, and referred to in this paper as algal xylan. It contained 93.3% DM and (% DM): minerals, 22.8; sulphate, 11.7. Sulphate was formed from sulphuric acid added during processing. The sulphate determinations on the above products were performed by Inra, Nantes (Lahaye and Axelos, 1993). Resistant starch was provided by Inra (Nantes), and consisted of retrograded high amylose maize starch (Faisant et al, 1993).

### Fermentation system

The semi-continuous culture system used in this experiment was adapted from the 'Rusitec' designed by Czerkawski and Breckenridge (1977) for rumen fermentation studies, consisting of four independent 1 L capacity vessels. Fresh faecal samples were collected from two female subjects referenced as subjects A and B. Methane was never detected in the exhaled breath of subject A, whereas subject B was a methane excretor, but only exhaled moderate levels of methane (about 20 ppm). Freshly voided faeces were stored under anaerobic conditions and processed within 48 h. All handling procedures were done under anaerobic conditions (under a gas phase of 100%  $N_2$ ).

For each vessel, 100 g faeces were homogenized in 500 mL  $CO_2$ -saturated mineral solution similar to that described by Michel et al (1996), except for  $Na_2SO_4$ , which was reduced by 50% (0.05 g/L). The faecal slurries were then strained through four layers of surgical gauze, and the strained fluid was introduced into the fermentation vessels. The solid residues were placed into nylon bags (160 × 80 mm, 150  $\mu$ m pore size) together with 5 g cut pre-fermented straw. This

bag was introduced into the vessel and remained there throughout the experiment in order to permit bacterial attachment. The volume in the vessel was then adjusted to 1 L with a nutritive basal medium. This contained the mineral solution described above, together with a mixture of organic compounds (Sigma, France) (g/L): porcine gastric mucin, 2; pancreatin, 1; casein, 2; cysteine, 0.2; haemin, 0.01; peptone, 0.01; bile salts (50% sodium cholate), 0.05; urea, 0.18; xylan, 0.5; pectin, 0.5; amylopectin, 0.5; arabinogalactan, 0.5; lintner starch, 0.5 and vitamins (mg/L): riboflavin, 0.5; biotin, 0.012; cyanocobalamin, 0.005; folic acid, 0.012; nicotinic acid, pyridoxine HCl, thiamine HCl and pantothenate, 1 mg/L each.

Each vessel was infused with the basal medium (BM) at an approximate rate of 1 L/d. When *Ulva* was tested, nylon bags containing 15 g *Ulva* were introduced into the vessels and removed 48 h later. The bags were washed with the mineral solution, and the washings were put back into the fermentation vessels. When algal xylan and resistant starch were used to supplement *Ulva*, they were injected into the vessel twice per day as a suspension in distilled water (3 g/20 mL and 2.5 g/20 mL for algal xylan and resistant starch, respectively). When ulvan was tested, the substrate was solubilized in distilled water (2.5 g/20 mL) and injected twice daily into the fermenters. Sodium molybdate and 2-bromoethanesulfonic acid, when necessary, were introduced into the basal medium and infused continuously.

## Experiments

Four experiments (studies 1 to 4) were conducted to study *Ulva* fermentation and sulphate metabolism and one (study 5) to study ulvan fermentation and sulphate metabolism. The inoculum from subject A (non-methanogenic) was used in studies 1, 2 and 5 and the inoculum from subject B was used in studies 3 and 4. The experiments were conducted according to the following experimental design:

**Study 1:** fermentation of the basal culture medium without (one vessel) or with addition of *Ulva* (one vessel) was followed for 12 days.

**Study 2:** the aim in this experiment was to concentrate on sulphate metabolism and its effect on reductive acetogenesis. Two vessels were first

adapted to *Ulva* for 10 days, then one of the vessels received sodium molybdate (20 mM), and the experiment was run for a further 10 days.

**Study 3:** *Ulva* fermentation and sulphate metabolism were studied for 7 days in the presence (two vessels) or absence (two vessels) of the methanogenesis inhibitor 2-bromoethanesulfonic acid (BES) (20 mM), since the inoculum B was putatively methanogenic. The former vessels (*Ulva* alone) also served as a control for study 4.

**Study 4:** the effect of *Ulva* supplementation with algal xylan (two vessels) and resistant starch (two vessels) on the pattern of fermentation and sulphate metabolism was investigated for 7 days. One vessel of each group received sodium molybdate (15 mM) from the beginning of the incubation.

**Study 5:** fermentation and sulphate metabolism in the basal culture medium alone (two vessels) or supplemented with ulvan (two vessels) were studied for 7 days. One vessel from each group received sodium molybdate as indicated above.

## Bacteriological and chemical analyses

### *Bacterial enumerations*

Total anaerobes, methanogenic archaea, sulphate-reducing and acetogenic bacteria were enumerated in experiments 2 and 3 in the original faecal samples (donors A and B, respectively) and in the fermentation vessels at the end of the incubations, following the methods described by Doré et al (1995a) and Bernalier et al (1996a).

### *Chemical analyses on spent culture media*

Effluents from the fermenters were collected daily. SCFA were analysed by capillary GC after conversion to tertiary butyldimethylsilyl derivatives (Richardson et al, 1989). Ammonia nitrogen ( $\text{NH}_3\text{-N}$ ) and protein nitrogen (protein-N) were determined on three consecutive days at the end of studies 1, 2 and 4.  $\text{NH}_3\text{-N}$  was measured by titration after displacement by magnesium oxide (0.1 M), and protein-N was precipitated by trichloroacetic acid (10% final concentration), centrifuged and determined by the Kjeldahl method. Sulphate was determined at intervals in

all experiments except for study 1. Sulphate was assayed by turbidimetry after treatment with TCA and in the presence of  $\text{BaCl}_2$  and polyethylene glycol (Bo Sörbo, 1987).

### ***Analyses on the liquid phase of the fermenters***

Samples were taken from the fermenters every morning just before the change of feed bag of *Ulva*, or just before the injection of ulvan. On these samples, pH was recorded daily and sulphides were determined at intervals in studies 2 and 3 and daily in studies 4 and 5. Sulphides were assayed by the colorimetric method of Cline (1969) after precipitation of sulphides in 10% (wt/vol) zinc acetate solution (Gibson et al, 1988b). In addition, on two different days after adaptation, pH was recorded at different times up to 7 h after feeding the substrates.

### ***Collection and analysis of gases***

Fermentation gases corresponding to each vessel were collected daily in a glass cylinder and volume was measured by liquid displacement. Except for studies 1 and 2, the gas was previously bubbled through zinc acetate (10% wt/vol) to trap any gaseous sulphide. Samples of gas were then analysed for  $\text{H}_2$ ,  $\text{CH}_4$  and  $\text{CO}_2$  by GC (De Graeve et al, 1994) and trapped sulphide was determined as above.

### ***Measurement of reductive acetogenic activity by $^{13}\text{C}$ NMR***

At the end of studies 2 and 3 the microbiotas from each vessel were collected by centrifugation (27 000 g for 30 min at 4 °C) under  $\text{N}_2$  gas phase. Aliquots were incubated for 16 h in 120 mL penicillin flasks in the presence of  $\text{NaH}^{13}\text{CO}_3$  with  $\text{H}_2$  as gas phase. The effect of sulphate addition was assessed by adding 7 mM  $\text{Na}_2\text{SO}_4$  to half of the flasks. Incorporation of  $^{13}\text{CO}_2$  into acetate was measured by  $^{13}\text{C}$ -NMR as described by De Graeve et al (1994).

### ***Statistical analysis***

Experimental values were compared by ANOVA and with the Newman-Keuls multiple range test (STATITCF software; ITCF, Paris).

## **RESULTS**

### **Bacterial enumeration**

In the original faeces, the number of total anaerobes and of reductive acetogens was lower with donor B while those of sulphate-reducing bacteria (SRB) were similar for both individuals (table I). In agreement with breath tests, methanogens were not detectable in faeces from donor A, and their numbers were just significant in those from donor B.

In the fermentation vessels, despite the ten-fold dilution of faeces, the number of total anaerobes, SRB and acetogenic bacteria remained close to that of the original faeces, with both inocula. However, the number of methanogens from donor B was greatly decreased compared to the original faeces. Addition of sodium molybdate lowered the SRB population and that of BES practically eliminated the methanogens (table I).

### **End products of fermentation**

Results concerning the production of SCFA in the presence of *Ulva* that were obtained in studies 1 and 2 with inoculum A did not show any difference with those obtained in study 3 with inoculum B. The mean production after 5 days adaptation was  $70.5 \pm 2.1$  and  $70.9 \pm 2.0$  mmol/day for donors A and B, respectively. Therefore, data obtained with both inocula were pooled.

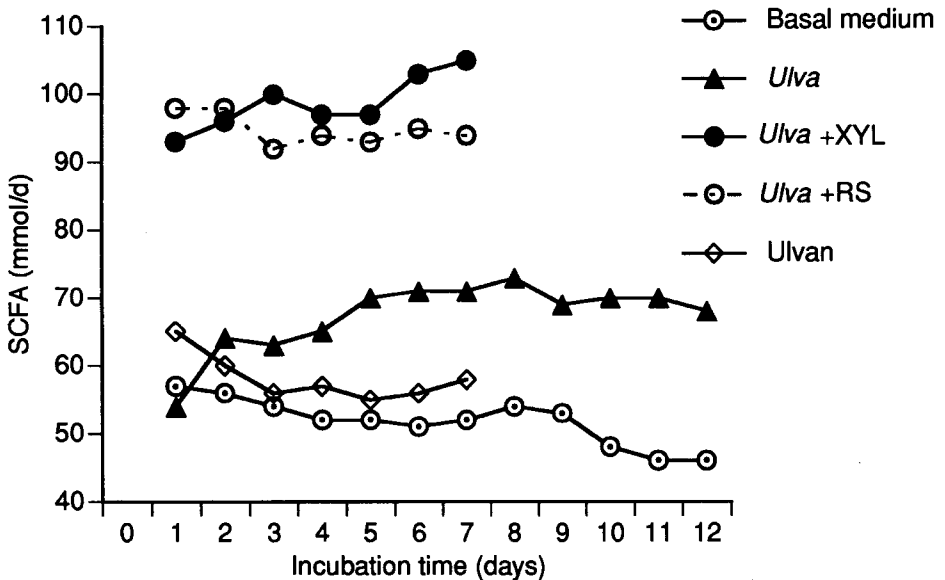
### ***Effect of adaptation on SCFA production***

Daily production of SCFA during 12 days adaptation to the basal medium was steady from day 4 to day 9, before decreasing by 10% (fig 1). During the same period, *Ulva* addition resulted in a gradual rise in SCFA, which levelled off after day 5 of the incubation, showing that about 5 days were required for full adaptation of the flora to

**Table I.** Enumeration of total viable anaerobes and H<sub>2</sub>-utilizing microorganisms in original faeces and in fermenter contents at the end of incubation.

	Original counts Log N/g faeces (wet)	Final counts Log N/mL vessel content	
		Ulva	Ulva + Mo <sup>a</sup>
<b>Inoculum A</b>			
Experiment 2			
Total anaerobes	11.6	9.4	10.1
Sulphate reducing bacteria	8.9	10.1	6.2
Acetogenic bacteria	6.4	6.4	6.4
Methanogenic archaea	< 2.0	< 2.0	< 2.0
		<i>Ulva</i>	<i>Ulva + BES</i> <sup>b</sup>
<b>Inoculum B</b>			
Experiment 3			
Total anaerobes	10.6	10.7	10.2
Sulphate reducing bacteria	8.7	8.9	8.2
Acetogenic bacteria	4.9	4.4	4.8
Methanogenic archaea	7.5	4.4	< 2.0

<sup>a</sup> Mo: sodium molybdate; <sup>b</sup> BES: 2-bromoethane-sulfonic acid.

**Fig 1.** Influence of adaptation on daily SCFA production with the basal medium, *Ulva*, *Ulva* supplemented with algal xylan (*Ulva* + XYL), *Ulva* supplemented with resistant starch (*Ulva* + RS) and ulvan.

*Ulva* (fig 1). Consequently, the durations of studies 3-5 were reduced to 7 days.

Supplementation of *Ulva* by algal xylan (XYL) and resistant starch (RS) greatly increased total SCFA production from the first day of incubation. Then, it remained nearly constant with resistant starch and increased slightly with xylan (fig 1). When ulvan was the substrate, SCFA production first decreased by 15% from day 1 to day 3 and then stabilized at a level only slightly higher than that of the basal medium alone (fig 1).

### Balance of end products

The balance of end products was calculated as the mean of the production on days 5, 6 and 7 (table II). Compared to the basal medium, *Ulva* addition significantly increased all SCFAs and had only a small effect on their molar proportion (table II): there was a small decrease in acetate ratios and a slight but significant rise in valerate and the branched chain fatty acids (BCFA)

isobutyrate and isovalerate. Calculation of SCFA production owing to *Ulva* alone (*Ulva* vessels minus basal medium vessels) showed that (weight by weight), 9% of the crude product or 16.6% of its organic matter were recovered as SCFA.

The increase in SCFA effected by carbohydrate supplementation was significantly more marked with algal xylan than with resistant starch. Compared to *Ulva* alone, algal xylan elevated the molar proportion of propionate and lowered that of butyrate, whereas resistant starch slightly increased acetate. Both carbohydrates decreased the relative proportions of isovalerate and valerate (table II). In addition, resistant starch resulted in the formation of some longer chain fatty acids such as caproic and heptanoic acids (1.7 and 0.7 mmol/day, respectively). SCFA formed from algal xylan and resistant starch alone (carbohydrate supplemented vessels minus *Ulva* vessels) amounted to (weight by weight) 34 and 37% of the crude products, and to 47.9 and 41.4% of their organic matters, respectively.

**Table II.** Daily end products of fermentation from the basal medium (BM) and after addition of *Ulva*, *Ulva* + algal xylan (+ XYL), *Ulva* + resistant starch (+ RS) and ulvan, A 20.

	BM	<i>Ulva</i>	+ XYL	+RS	<i>Ulvan</i>	Pooled SE
Short-chain fatty acids	51.6 <sup>c</sup>	70.4 <sup>c</sup>	101.4 <sup>a</sup>	92.6 <sup>b</sup>	56.4 <sup>d</sup>	2.1
Total (mmol/day)						
Molar proportion (%)						
Acetic	61.0 <sup>a</sup>	57.9 <sup>b</sup>	58.1 <sup>b</sup>	60.4 <sup>a</sup>	62.0 <sup>a</sup>	0.8
Propionic	20.5 <sup>b</sup>	20.9 <sup>b</sup>	26.3 <sup>a</sup>	19.9 <sup>b</sup>	19.1 <sup>b</sup>	0.8
Butyric	10.7 <sup>a</sup>	11.5 <sup>a</sup>	9.0 <sup>b</sup>	11.3 <sup>a</sup>	10.9 <sup>a</sup>	0.5
Isobutyric	2.1 <sup>b</sup>	2.8 <sup>a</sup>	2.0 <sup>b</sup>	2.6 <sup>a</sup>	2.2 <sup>b</sup>	0.1
Valeric	3.9 <sup>b</sup>	4.5 <sup>a</sup>	3.0 <sup>c</sup>	4.0 <sup>b</sup>	3.8 <sup>b</sup>	0.2
Isovaleric	1.9 <sup>b</sup>	2.5 <sup>a</sup>	1.5 <sup>c</sup>	1.9 <sup>b</sup>	1.9 <sup>b</sup>	0.2
Carbon dioxide (mmol/day)	8.2 <sup>e</sup>	17.5 <sup>c</sup>	28.0 <sup>b</sup>	32.4 <sup>a</sup>	12.4 <sup>d</sup>	2.3
Ammonia-N (mg/day)	412 <sup>c</sup>	563 <sup>a</sup>	500 <sup>b</sup>	487 <sup>b</sup>	—	29
Protein-N (mg/day)	133 <sup>c</sup>	175 <sup>b</sup>	276 <sup>a</sup>	294 <sup>a</sup>	—	13
pH	6.90 <sup>a</sup>	6.86 <sup>b</sup>	6.74 <sup>c</sup>	6.76 <sup>c</sup>	6.84 <sup>b</sup>	0.02

Results are means of days 5, 6, 7.

$n = 6$  for BM, 12 for *Ulva*, 3 for *Ulva*, + XYL, + RS and *ulvan*.

<sup>a, b, c, d, e</sup> : means within a row, values with different superscripts were significantly different at  $P < 0.05$ .

Compared to the basal medium, the addition of ulvan increased slightly (< 10%), although significantly, total SCFA production, and this increase was entirely due to a rise in acetate (table II). The amount of SCFA produced from ulvan (ulvan vessel minus basal medium vessel) represented only 6.4% of crude ulvan and 8.9% of its organic matter.

Carbon dioxide (CO<sub>2</sub>) was the main fermentation gas produced. Compared to the basal medium, addition of *Ulva* and its further supplementation with algal xylan or resistant starch greatly increased the volume of CO<sub>2</sub> released (from about 200 mL up to 800 mL/day). Ulvan addition also resulted in a significant increase in CO<sub>2</sub> (+ 34%) compared to the basal medium. Hydrogen was detected at very low levels except on the last two days of incubation in the presence of resistant starch (mean: 2.2 mmol/day), and following sodium molybdate addition (see below). Only trace amounts of methane were detected in the vessels from studies 3 and 4, which were inoculated with faeces from donor B. Therefore, the addition of BES in study 3 had no effect at all on the fermentation end products from *Ulva* (results not shown).

Both protein- and ammonia-N output were increased by *Ulva* addition. Algal xylan and resistant starch supplementation of *Ulva* increased protein-N but decreased ammonia-N (table II).

The initial (before feeding) pH of the fermenters receiving the basal medium, *Ulva* alone and ulvan were not significantly different and remained near neutral (6.8-6.9) (table II). When both carbohydrates were added, the initial pH values were significantly lower (- 0.1 unit). After feeding, the pH rose briefly with *Ulva*, decreased within 2 h with xylan and remained stable with resistant starch (fig 2).

## Sulphate metabolism and the effect of sodium molybdate

### *Sulphate metabolism in the absence of molybdate*

The basal medium contained very low levels of sulphate (0.35 mmol/L). Nevertheless, sulphides were detected in culture vessels at a higher concentration than that of sulphate, demonstrating that some sulphides must have originated from other sources.

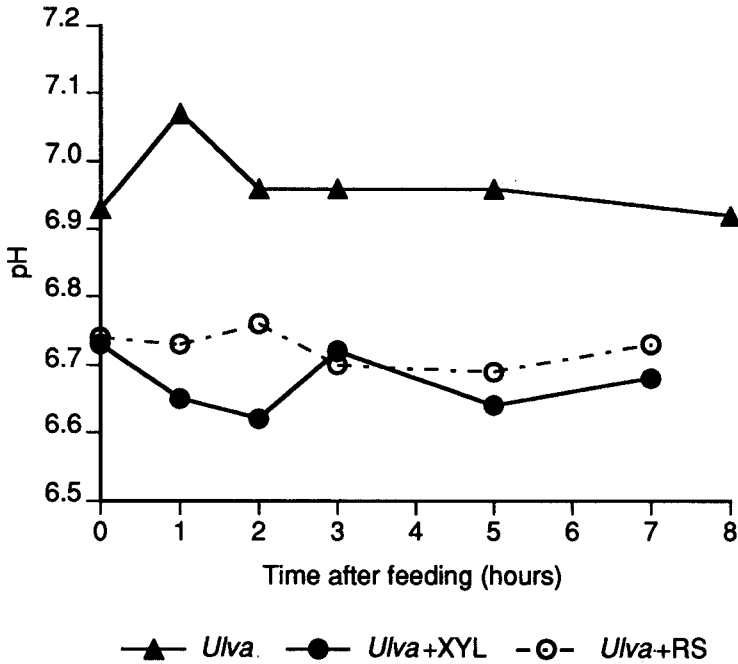
*Ulva* feeding increased the daily sulphate input by 15 mmol. However, irrespective of the inoculum, sulphate recovery in the effluent was low, while sulphide concentrations in the vessels rose to about 6 mmol/L (6.0 ± 0.6 for inoculum A, and 6.7 ± 0.6 for inoculum B). In table III, the results of both inocula were pooled.

*Ulva* supplementation with resistant starch did not change the sulphate input, whereas algal xylan added 6.8 mmol/day. It can be observed from figure 3 that with resistant starch, sulphate disappeared from the culture effluents within the first 3 days of incubation, whereas with algal xylan, it increased up to day 3, before declining. With both carbohydrates, sulphide concentrations in the vessels rose up to day 4, and then remained almost constant (6 to 7 mmol/L) (fig 3). Finally, on the last days of incubation, the sulphide concentrations were not significantly different for all vessels fed *Ulva* (table III).

Some gaseous sulphide was produced from the vessels fed *Ulva*, but the amounts were low (mean values for the three last days: 0.56 ± 0.05, 0.95 ± 0.10 and 0.68 ± 0.03 mmol/day for *Ulva* alone, + RS and + XYL, respectively).

Ulvan addition supplied daily a total of 7.4 mmol sulphate. Some sulphate was recovered in the culture effluents but sulphide concentrations in the fermenters remained similar to those of the basal medium (table III).





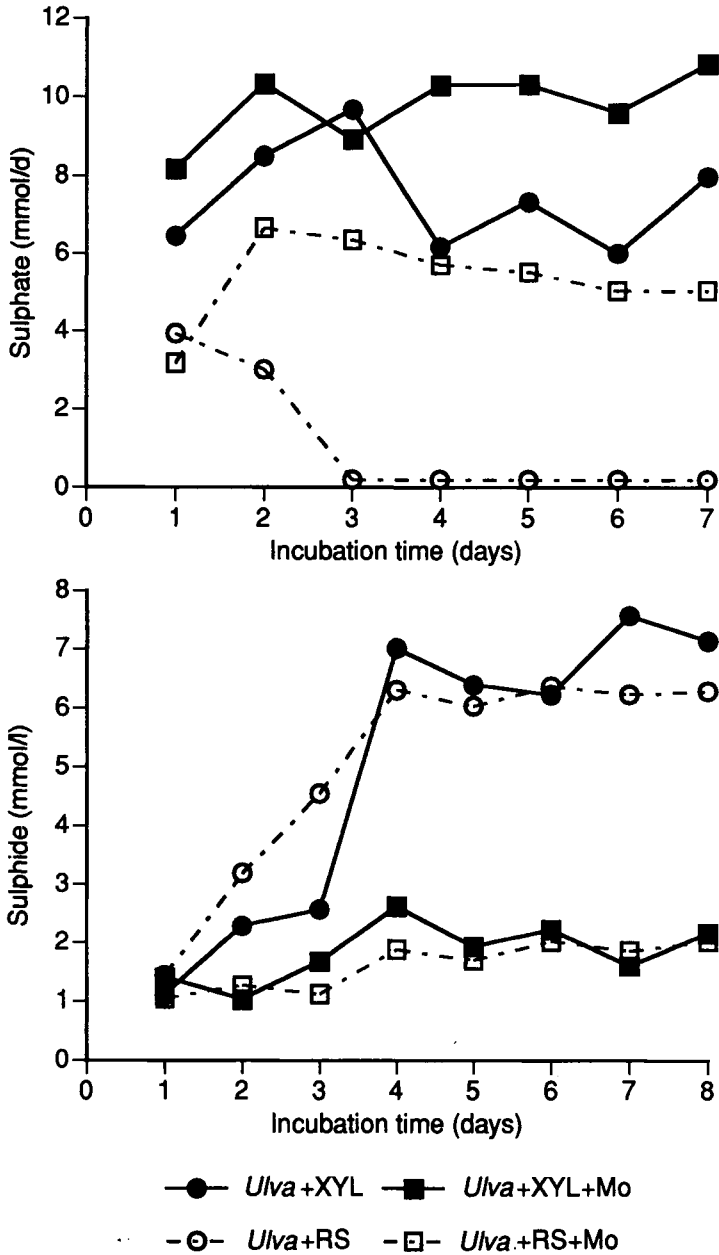
**Fig 2.** pH measurements in the fermenters after feeding *Ulva*, *Ulva* supplemented with algal xylan (*Ulva* + XYL) and *Ulva* supplemented with resistant starch (*Ulva* + RS).

**Table III.** Recovery of sulphate in the effluent and sulphide concentration in the vessels in the presence and absence of sodium molybdate (Mo).

	Sulphate recovery (mmol/day)		Sulphide concentration <sup>a</sup> (mmol/L)	
	-Mo	+Mo	-Mo	+Mo
Basal medium	nd <sup>d</sup>	T <sup>e</sup>	1.6 ± 0.1	1.2 ± 0.1
<i>Ulva</i>	1.7 ± 1.4	8.1 ± 0.3	6.3 ± 0.7	1.4 ± 0.1
<i>Ulva</i> + XYL <sup>b</sup>	7.1 ± 1.0	10.3 ± 0.7	7.0 ± 0.7	2.0 ± 0.3
<i>Ulva</i> + RS <sup>c</sup>	T <sup>e</sup>	5.2 ± 0.3	6.3 ± 0.1	2.0 ± 0.1
Ulvan	2.0 ± 0.2	2.6 ± 0.6	1.5 ± 0.2	1.1 ± 0.2

Results are means ± standard errors of the three last days of experiments.

<sup>a</sup> Before feeding the substrates; <sup>b</sup> XYL, algal xylan; <sup>c</sup> RS, resistant starch; <sup>d</sup> nd, not detectable; <sup>e</sup> T, trace amounts (< 0.3 mmol).



**Fig 3.** Influence of adaptation on daily sulphate output and sulphide concentrations in fermenters fed *Ulva* supplemented with algal xylan (*Ulva* + XYL) and *Ulva* supplemented with resistant starch (*Ulva* + RS) in the presence and absence of sodium molybdate (+ Mo).

### **Effect of molybdate addition on sulphate metabolism**

With the basal medium and with ulvan, molybdate had only a slight depressing effect on sulphide concentrations ( $-0.4$  mmol/L). Conversely, in all the vessels fed *Ulva*, molybdate significantly increased sulphate output and strongly decreased sulphide concentrations. However, the increase in sulphate output was less marked in the presence of algal xylan (fig 3, table III).

### **Effect of molybdate addition on end products of fermentation**

Whatever the treatments, total SCFA production was decreased by molybdate. This was generally due to a reduction in acetate, except with xylan, where propionate was reduced by 30% (table IV). In all the vessels fed *Ulva*, molybdate addition resulted in production of formate and hydrogen (fig 4). With *Ulva* alone, these productions were low and transient, whereas they were more abundant and lasted longer with addition of carbohydrates, especially with resistant starch (fig 4).

### **Reductive acetogenesis of the microflora adapted to *Ulva***

Despite adaptation of the microflora to *Ulva*, which was rich in sulphate, synthesis of

$^{13}\text{C}$ -acetate from  $^{13}\text{CO}_2$  was intense in the absence of added sulphate (table V). In both cases the ratio of double-labelled acetate to total labelled acetate represented about 40%, and on average, the labelling was equally distributed among the methyl and carboxyl groups of acetate.

Adaptation of the microflora to molybdate (study 2) reduced both total acetate production by the culture and the proportion of labelled acetate in total acetate (table V).

Addition of sodium sulphate to the bath cultures reduced total acetate and the synthesis of acetate from  $^{13}\text{CO}_2$  in study 2 but not in study 3. Here, one of the vessels that showed a high  $^{13}\text{CO}_2$  incorporation into acetate was not negatively affected by sulphate addition in contrast with the other two. This explains the particularly high values of standard errors (table V).

## **DISCUSSION**

### **Fermentation pattern**

In this study, we used continuous rather than batch cultures because we hypothesized that adaptation of the colonic bacteria to the seaweed *Ulva* and its main soluble constituent ulvan was necessary to optimize their fermentation, since these substrates are not routinely part of the diet. The results we obtained on SCFA production from *Ulva* do not show strong adaptative effects (fig 1).

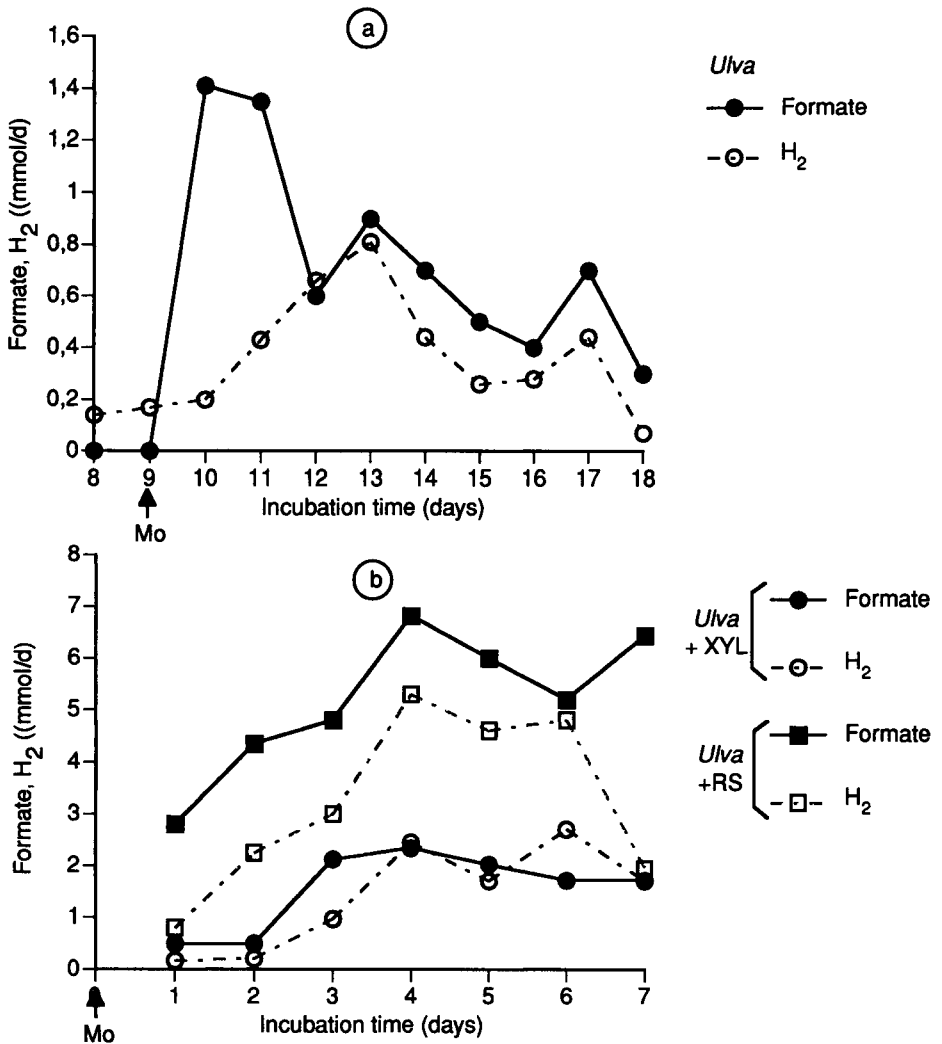
**Table IV.** Changes (+ or -) of short chain fatty acid production induced by sodium molybdate (Mo) (mmol/day) (means  $\pm$  SE of d5, 6 and 7 after Mo addition).

	BM	Ulva	+ XYL	+ RS	Ulvan
Total SCFA	$-8.9 \pm 1.9$	$-5.7 \pm 1.7$	$-9.5 \pm 3.7$	$-16.6 \pm 2.2$	$-4.9 \pm 1.7$
Acetate	$-7.8 \pm 0.8$	$-11.0 \pm 1.0$	nsc <sup>a</sup>	$-16.1 \pm 2.2$	$-6.1 \pm 1.0$
Propionate	nsc <sup>a</sup>	$+3.6 \pm 1.5$	$-8.6 \pm 1.1$	$-3.5 \pm 1.5$	nsc <sup>a</sup>
Butyrate	nsc <sup>a</sup>	nsc <sup>a</sup>	$+1.7 \pm 0.7$	$+2.7 \pm 1.1$	$+0.8 \pm 0.2$

<sup>a</sup> nsc: no significant change.

The first period of adaptation may have been due to enzyme induction, which is a comparatively rapid process (Salyers, 1985; Macfarlane et al, 1990). Then the number of bacterial species able to degrade *Ulva* may have slightly increased. This slight adaptation of the microflora to *Ulva* was

not shown with ulvan. On the contrary, inhibitory effects on SCFA production seemed to occur from day 1 to day 3 of the incubation. This observation is consistent with the partial inhibitory effect of ulvan over cellulase activity that has been recently detected (Bobin-Dubigeon, 1996).



**Fig 4.** Effect of sodium molybdate on formate and hydrogen (H<sub>2</sub>) production with *Ulva*, a and b, *Ulva* supplemented with algal xylan (*Ulva* + XYL) and *Ulva* supplemented with resistant starch (*Ulva* + RS).

**Table V.** Total acetate and  $^{13}\text{C}$ -acetate formation from  $^{13}\text{CO}_2$  in batch incubations of *Ulva* adapted flora.

Acetate	Inoculum A, study 2			Inoculum B, study 3	
	-Mo $-\text{SO}_4^{2-}$	+Mo $-\text{SO}_4^{2-}$	-Mo $-\text{SO}_4^{2-}$	$-\text{SO}_4^{2-}$	+ $\text{SO}_4^{2-}$
Total (mM)	43.4 ± 1.6	21.2 ± 0.6	33.6 ± 0.4	39.9 ± 2.5	38.4 ± 4.3
Total labelled (mM)	12.9 ± 2.0	4.1 ± 1.0	7.5 ± 1.0	14.7 ± 2.8	11.4 ± 4.5
Double labelled					
% total labelled	44.0 ± 1.4	42.5 ± 1.9	38.7 ± 0.6	43.5 ± 3.0	41.0 ± 8.3

+Mo, the flora had been previously incubated in the presence of sodium molybdate and 10 mM sodium molybdate were added to the batch cultures. + $\text{SO}_4^{2-}$ , 7 mM sodium sulphate were added to the batch cultures.

*Ulva* was fermented to a very limited extent compared to algal xylan and resistant starch, which provided three times more SCFA per gramme of organic matter. In addition, the small amounts of SCFA formed from *Ulva* did not appear to originate from its soluble carbohydrates: ulvan. These data support the previous findings of Bobin-Dubigeon et al (1996) who, using batch culture, showed that insoluble fibres contained in *Ulva* were more fermentable than the soluble ones (ulvan). This resistance was mainly attributed to the particular chemical structure (linkage and sequence of sugars) of these soluble polymers (Bobin-Dubigeon et al, 1996) and to the lack of the specific depolymerases in colonic microorganisms (Michel and Macfarlane, 1996).

Addition of *Ulva* to the basal medium increased the relative proportion of the BCFA, isobutyrate and isovalerate. Similar observations were made in batch cultures (Bobin-Dubigeon et al, 1996). These BCFA are generated almost exclusively from amino acid fermentation, especially from valine and leucine, and are therefore good markers of protein breakdown (Macfarlane and Cummings, 1991). From the results of production of SCFA from casein and bovine serum albumin obtained in batch-cultures, Mac-

farlane et al (1992) assumed that approximately 30% by weight of the protein broken down is converted to SCFA and that BCFA constitute 20% of these SCFA. These data enabled us to estimate the proteinaceous materials that were broken down during our incubations. The calculated values were 2.4 g/day for the basal medium alone and 2 g/day for *Ulva* alone. This would mean that all the proteinaceous materials of the basal medium and about half those of *Ulva* were degraded which is in line with the amount of ammonia produced. In vivo, it is not known whether and to what extent *Ulva* proteins are absorbed in the upper part of the intestine. Their contribution to the formation of SCFA or of ammonia in the large intestine cannot therefore be estimated.

Yields of SCFA obtained from the fermentation of algal xylan and resistant starch were relatively high (48 and 41% organic matter, respectively) and comparable with the figures given for cell wall polysaccharides (35-54%) by Cummings (1995). These substrates provided energy for microbial growth as shown by the clear-cut increase in protein formation following their addition. We can estimate that 2.3 and 2.6 g microbial protein-N were produced by 100 g OM fermented for algal xylan and resistant starch,

respectively. This corresponds approximately to an efficiency of microbial synthesis of 30% (weight by weight), a value that is comparable with that calculated by Cummings (1984), from excretion of faecal bacterial mass. The decrease in ammonia observed with these polysaccharides could result either from inhibition of amino acid deamination, or from a greater ammonia uptake for microbial protein synthesis. The latter was more likely under our conditions because the absolute levels of BCFA did not change with carbohydrate supplementation. Ammonia formation has a number of possible harmful health implications (Macfarlane and Cummings, 1991), therefore, if part of *Ulva* proteins reach the large intestine, supplementing this product with such polysaccharides should be beneficial for the host.

Algal xylan and resistant starch showed different fermentation patterns. Resistant starch (retrograded amylo maize starch) had a slow rate of fermentation, as shown by the stability of the pH after feeding. These characteristics were already observed when we compared the same resistant starch to lactulose (Doré et al, 1995b). Algal xylan fermented rapidly, induced a pH decrease shortly after feeding, and produced high proportions of propionic acid, thus corroborating previous observations of Lahaye et al (1993).

#### **Metabolism of sulphate from *Ulva* and possible interactions with methanogenesis and acetogenesis**

Our study clearly showed the importance of sulphide production in all the vessels fed *Ulva*. About 40% of *Ulva* sulphate was dissimilated to sulphide. The effect of the inhibitor, sodium molybdate (Mo), which greatly decreased sulphide concentration and increased sulphate output from the vessels, further demonstrated the high level of activity of sulphate-reducing bacteria in the

presence of *Ulva*. The formation of low levels of sulphide, either in the basal medium, or in Mo-supplemented vessels originated most probably from microbial degradation of the sulphur amino acids, cysteine and methionine in which sulphur exists in its most reduced state. In the rumen, cysteine is known to be degraded into  $\text{NH}_3$ , pyruvate and  $\text{H}_2\text{S}$  in the presence of sulphhydrylase and feeding methionine to sheep increased the rumen sulphide pool (Doyle and Moir, 1979). We had first expected that supplementation of *Ulva* with fermentable carbohydrates such as resistant starch would reduce sulphide concentrations by decreasing the pH and/or by stimulating assimilatory sulphate reduction in relation to the increase in biomass synthesis. Our results did not support this prediction. First, probably because of the high concentration of ammonia, the pH did not decrease below 6.7 and dissimilatory sulphate reduction was shown to be reduced only below pH 6.5 (Gibson et al, 1990). Further, the rise in biomass synthesis did not support high levels of sulphate incorporation. If we assume that the value of S/N ratio in the colonic flora is similar to that reported for rumen microorganisms (S/N = 0.06) (Durand and Komisarczuk, 1988), we can approximate an increase of S incorporation into biomass of 0.2-0.4 mmol, corresponding to the 200 mg protein-N induced by resistant starch. This value is negligible compared to the observed sulphide concentrations.

Our results showing that sulphide production from ulvan was negligible confirm the recent observations of Michel et al (1995). These authors concluded that resistance to desulphation appeared to be the limiting factor in ulvan sulphate metabolism by SRB. However, our particular ulvan preparation contained some free sulphate (about 30%) (Lahaye, personal communication) which was recovered in the culture effluents in the presence as well as in absence of Mo. This would indicate an

inhibitory effect of ulvan itself on dissimilatory sulphate reduction.

In our studies, inhibition of sulphide reduction by Mo resulted in release of formate and hydrogen. Gibson et al (1988a) also observed that inhibition of SRB by Mo resulted in accumulation of hydrogen in large quantities. In our experiments, however, the production of hydrogen and formate were greater in the presence of resistant starch than with *Ulva* alone, or when supplemented with algal xylan, despite similar levels of sulphide production in the absence of Mo. This indicates that electron donors for sulphate reduction varied depending on the nature of the polysaccharides being fermented. Low molecular weight organic acids such as lactate, propionate or acetate can be used as electron donors in addition to H<sub>2</sub> and formate (Gibson, 1990). Sodium molybdate was not entirely specific against sulphate reduction. Although it did not apparently decrease the total number of anaerobes, it always reduced, whatever the treatment, production of SCFA. This reduction was most apparent with resistant starch (-18%). In this case, inhibition of acetate production might have been the consequence of hydrogen accumulation, which is known to inhibit regeneration of reduced electron carriers involved in the oxidation of pyruvate to CO<sub>2</sub> and acetyl-CoA in a number of bacterial species. This explanation is not valid, however, for the basal medium, or for *Ulva*, where H<sub>2</sub> production was very low. Our results of <sup>13</sup>C<sub>2</sub>O<sub>2</sub> incorporation into acetate also clearly demonstrate an inhibitory effect of Mo on reductive acetogenesis. Molybdate being a competitive inhibitor, should be added at the equivalent level of ambient sulphate dissolved in the system. A level of 20 mM molybdate is routinely employed in human faecal incubations (Gibson et al, 1988a; Strocchi et al, 1993) without notable inhibitory effects. It is possible that some microbial strains are more susceptible to Mo in continuous rather than in batch cultures. Some inhibitory effects of 20 mM Mo

on methane production have been recorded (Oremland and Capone, 1988). Mo is known to form molybdosulphide complexes which can bind available sulphide ions and cause a deficiency in sulphur availability.

The number of sulphate-reducing bacteria (SRB) was relatively high and similar in both original faeces, though donor B was moderately methanogenic and harboured a significant number of methanogens in her faeces. This absence of a clear-cut inverse relationship between faecal SRB and methanogens is in agreement with the recent results of Doré et al (1995a). However, after adaptation to *Ulva* in the fermenters inoculated with the flora from donor B, the number of methanogens decreased and only trace amounts of CH<sub>4</sub> were produced. This inhibition of methanogenesis was, most probably, related to the high sulphate content of *Ulva* which led to competitive exclusion of methanogens by SRB, as previously observed by Gibson et al (1988a). Here, this exclusion was probably facilitated by the fact that the inoculum was provided by a low CH<sub>4</sub> excretor and harboured a moderate number of methanogens. Contrary to methanogenesis, neither the concentration of acetogens nor the reductive acetogenic activity were affected by the adaptation to *Ulva*. Pure cultures of acetogenic bacteria from the human colon have been shown recently to be extremely versatile with regard to their substrate utilization patterns (Bernalier et al, 1996b), which could facilitate their growth in the presence of active sulphate reducers. The addition of sulphate at a level of 7 mM which is what could be expected in the colon of human subjects consuming about 15 g dry *Ulva*, counteracted reductive acetogenesis in one experiment out of two, demonstrating that SRB present in some individual inocula can compete with acetogenic bacteria for H<sub>2</sub>. This is consistent with previous observations showing fluctuating responses to sulphate addition (Bernalier et al, 1996a).

In conclusion, we confirmed that *Ulva*, and particularly its soluble polymer, ulvan, are to a large extent resistant to fermentation, and we have shown that adaptation of the microbiota occurs to a very limited degree. Our results also clearly demonstrate that *Ulva*, though producing low amounts of SCFA, stimulates sulphide formation by methanogenic and non-methanogenic microfloras, owing to its high free-sulphate content. Its supplementation with more fermentable polysaccharides, though probably beneficial for the host by decreasing ammonia, should not significantly reduce sulphide production. Therefore, it can be postulated that unless crude *Ulva* is previously processed in order to remove its free sulphate, its regular consumption at a level of 15 or 20 g of dry product daily could support high activities of sulphate reducing bacteria in the colon, which might interact with other microbial pathways of H<sub>2</sub> disposal and be detrimental to the health of the host.

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