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Bacterial monocultures, propionate, butyrate and H_2O_2 modulate the expression, secretion and structure of the fasting-induced adipose factor in gut epithelial cell lines

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Summary

Previous research showed that an intestinal microbial community represses the fasting-induced adipose factor (FIAF) in the gut epithelium, thereby increasing fat storage in the host. This study was designed to investigate the overall effect of different bacterial species and metabolites on FIAF in intestinal (Caco-2, HT-29 and HCT-116) and hepatic (HepG2) cancer cell lines. First, we showed that FIAF was present in different isoforms, and secreted as N-glycosylated proteins, exclusively at the basal side of the cell monolayer. Second, co-incubation of cell lines with bacterial monocultures and metabolites altered both FIAF production and isoform appearance. Propionate and/or butyrate treatment increased FIAF expression and cleavage in all tested cell lines. In contrast, different bacteria induced cell line-specific FIAF modulation. *Clostridium perfringens* induced FIAF isoform changes in Caco-2 cells. *Enterococcus faecalis* and *Bacteroides thetaiotaomicron* treatment resulted in cell line-specific FIAF increases, whereas *Escherichia coli* significantly decreased FIAF expression in HCT-116 cells. Treatment with H_2O_2 and peroxide-producing *E. faecalis* strains induced FIAF isoform changes in Caco-2 cells. Since bacteria and bacterial metabolites alter both FIAF production and isoform appearance, further investigation may reveal an important role for bacteria in FIAF-regulated physiological processes, such as cell differentiation and fat metabolism.

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Introduction

During recent years, the fasting-induced adipose factor (FIAF), also known as angiopoietin-like protein 4 (ANGPTL4), has been thoroughly investigated as a multifunctional signal protein, mainly expressed in the liver, adipose tissue, intestine, brain and thyroid tissue (Kersten *et al.*, 2009). FIAF is upregulated during fasting, hypoxia and adipocyte differentiation (Dutton and Trayhurn, 2008; Murata *et al.*, 2009). Furthermore, it is involved in the regulation of glucose tolerance (Xu *et al.*, 2005), angiogenesis (Le Jan *et al.*, 2003), lung and joint health (Murata *et al.*, 2009; Stapleton *et al.*, 2010), cancer development (Galaup *et al.*, 2006; Padua *et al.*, 2008; Verine *et al.*, 2010) and fat metabolism (Zandbergen *et al.*, 2006; Koliwad *et al.*, 2009; Lichtenstein and Kersten, 2010). Once secreted in the blood, FIAF inhibits the activity of lipoprotein lipase (LPL), an enzyme responsible for the conversion of triglycerides to monoglycerides and fatty acids from circulating lipoproteins (Mandard *et al.*, 2006; Lee *et al.*, 2009; Shan *et al.*, 2009). As a consequence, FIAF increases plasma triglyceride levels, and decreases the uptake of fatty acids and cholesterol into tissues (Lichtenstein *et al.*, 2007).

A particular feature of the intestinal FIAF gene is that its expression is strongly regulated by the presence of an intestinal microbial community (Bäckhed *et al.*, 2004; Fleissner *et al.*, 2010). FIAF expression is significantly repressed in mice with a normal intestinal microbial community compared with germ-free mice, and conventionalization of these germ-free animals with intestinal bacteria significantly decreased FIAF levels, resulting in enhanced fat storage and weight gain. Moreover, the elevated FIAF levels may, depending on the type of diet, protect germ-free mice against certain types of high-fat diet-induced obesity through induction of the peroxisome proliferator-activated receptor-γ co-activator-1α, thereby regulating genes involved in energy metabolism (Bäckhed *et al.*,

2007; Fleissner *et al*., 2010). Therefore, FIAF is considered as one of the key molecules involved in the microbial regulation of fat storage in mice. Yet, information on the type of microbial species and metabolites that modulate FIAF production by intestinal epithelial cells is very limited.

Gastrointestinal microorganisms may directly (by cell contact) or indirectly (by metabolite or secretion factors) modulate FIAF expression and production by intestinal epithelial cells. Common microbial metabolites such as short-chain fatty acids (SCFAs) and hydrogen peroxide (H_2O_2) have been shown to affect multiple intestinal cell signalling pathways (Hamer *et al*., 2008; Voltan *et al*., 2008). For example, *Lactobacillus crispatus*-derived H_2O_2 can act as signal-transducing molecule to activate the PPARγ pathway in the colon mucosa of mice and in the CMT-93 cell line (Voltan *et al*., 2008). Furthermore, butyrate can enhance apoptosis in the Caco-2 cell line via increased PPARγ transcription (Wächtershäuser and Stein, 2000), resulting in caspase-3 upregulation (Schwab *et al*., 2006). Butyrate has been shown to influence angiogenesis by Hif-1α regulation and activity (Zgouras *et al*., 2003). In addition, LPS treatment, which is a common cell wall component of Gram-negative bacteria, resulted in upregulation of Hif-1α in several types of carcinomas and colorectal cancer cell lines (Simiantonaki *et al*., 2008) and of FIAF in neuronal and adipocyte cells (Brown *et al*., 2009; Lu *et al*., 2010). As the transcription factors PPARγ and Hif-1α are both capable of regulating FIAF expression (Belanger *et al*., 2002; Mandard *et al*., 2004), it could be hypothesized that microbial modulation of gut epithelial FIAF expression is PPARγ or Hif-1α mediated.

In this study, we addressed the microbial modulation of gut epithelial FIAF production in three steps. First, we measured the endogenous expression and isoforms of FIAF in three different colorectal cancer cell lines in order to evaluate if these cell lines are a suitable model for studying FIAF production and secretion. Second, we co-incubated relevant gut microbes with Caco-2 cells to study whether microbial adhesion to gut epithelial cells is a prerequisite for FIAF processing. Finally, we investigated FIAF modulation by common bacterial metabolites in colorectal and hepatic cancer cell lines.

Results

Characterization of FIAF protein expression and secretion in different colon and hepatic cancer cell lines

Because it has been described that FIAF isoforms differ according to their origin and function (Ge *et al*., 2004; Mandard *et al*., 2004), we applied Western blotting and immunostaining with a specific antibody recognizing the various FIAF isoforms as a detection method because high-throughput methods such as ELISA (Xu *et al*., 2005; Kersten *et al*., 2009) or qPCR (Brown *et al*., 2006; Dutton and Trayhurn, 2008) do not discriminate between the different isoforms.

Figure 1 shows the different protein forms of FIAF expressed and secreted by the colon cancer cell lines Caco-2, HCT-116 and HT-29 and the hepatic cancer cell line, HepG2. In the insoluble fraction, which contains proteins predominantly derived from organelles and mem-

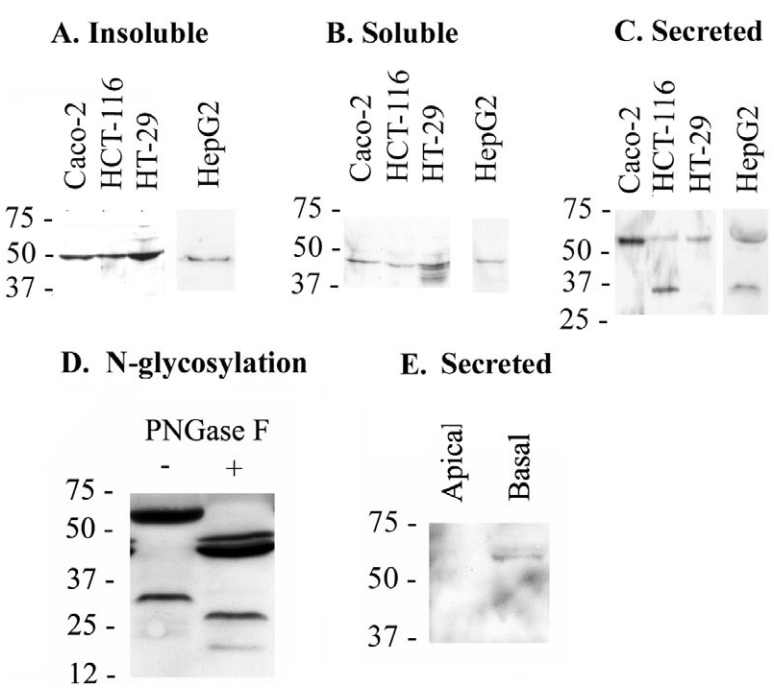

Fig. 1. FIAF in colorectal and hepatic cancer cell lines. The FIAF protein bands for (A) insoluble membrane and nucleus associated, (B) soluble cytoplasma and (C) secreted protein fractions of Caco-2, HCT-116, HT-29 cell and HepG2 cell lines. (D) PNGase F treatment of the secreted protein fraction indicate that the secreted ~67 and ~32 kDa forms are glycosylation products of the ~45 and ~41 kDa forms, and ~26 kDa and ~22 kDa forms of FIAF, respectively. (E) Secreted fraction in apical (Ap) and basal (Bas) compartments of differentiated Caco-2 cells seeded on Transwell plates.

branes, FIAF appeared at a MM of 50 kDa, as previously described by Kersten and colleagues (2000) (Fig. 1A). In the soluble fraction, which contains proteins predominantly from cytoplasmic origin, FIAF displayed a MM of approximately 41 kDa and 45 kDa (Fig. 1B). These forms have been reported as splice variants of FIAF according to the *Ensembl* database (http://www.ensembl.org). In the secreted fraction, FIAF appeared as a band of approximately 67 kDa (Fig. 1C), which has already been described as the N-glycosylated form (Ge *et al*., 2004; Yang *et al*., 2008). In addition, HCT-116 and HepG2 cells secreted a cleaved FIAF isoform of 32 kDa. PNGase F treatment of the secreted pool showed that the 67 and 32 kDa isoforms are N-glycosylated products of the 41–45 kDa and 26 kDa respectively (Fig. 1D).

The secreted FIAF protein fraction is of particular importance, because FIAF needs to enter the bloodstream to elicit a biological activity *in vivo*. Therefore, FIAF secretion was measured at the apical and basolateral side of differentiated Caco-2 cells, cultured on Transwell® plates. FIAF was released exclusively from the basal side of the cells (Fig. 1E), representing the side directed to the bloodstream.

Bacteria modulate FIAF expression in gut epithelial cell lines

The microbial species that contribute to the repression of the gut epithelial FIAF production, as was previously reported by Bäckhed and colleagues (2004), need further investigation. We therefore co-incubated colorectal cancer cells with monocultures of different bacterial species that belong to the main microbial phyla present in the gut. These species included *Bacteroides thetaiotaomicron* for the *Bacteroidetes* phylum, *Escherichia coli* for the γ-proteobacteria, *Lactobacillus brevis, Clostridium perfringens* and *Enterococcus faecalis* LMG 7937 for the *Firmicutes* phylum and *Bifidobacterium breve* for the *Actinobacteria*. In addition, we chose *B. thetaiotaomicron* and *E. faecalis* because FIAF modulation by these bacteria was already investigated previously. Subsequently, we chose two common probiotic strains (*B. breve* and *L. brevis*), a coliform (*E. coli*) and an opportunistic pathogen (*C. perfringens*).

In analogy with previous studies (Bernet *et al*., 1993; Lee and Puong, 2002), the co-incubation time of the colorectal epithelial cells and the bacteria was limited (2 h), since longer incubation resulted in detachment of the cells from the bottom of the culture flask. As a control for epithelial cell lysis, LDH analysis of the culture medium, before and after a 2 h incubation with bacteria, revealed no significant differences in LDH content (data not shown). Additionally, cleavage of PARP and LC3 as markers for apoptosis and autophagy, respectively, was evaluated in the soluble protein fraction of the bacteria-treated epithelial cells, but no cleavage was detected (data not shown). These control experiments assured us that the co-incubation experiments did not negatively affect the cell viability.

An overview of the effect of the specific monocultures on FIAF isoform and concentration in the different protein fractions of Caco-2, HT-29 and HCT-116 cells is presented in Table 1. Overall, no effect on expression levels could be found in the insoluble fraction with all tested species. In the soluble fraction, a shift from the 41 to 45 kDa form was observed towards the 50 kDa form after treatment with *C. perfringens* in Caco-2 cells (Fig. 2). Interestingly, this was only observed upon co-incubation with live *C. perfringens* bacteria. Incubation of Caco-2 cells with bacterium-free Dulbecco's modified Eagle's medium (DMEM), which had been pre-fermented with *C. perfringens,* or with heat-inactivated bacteria did not result in significant isoform changes (Fig. 2).

Moreover, Table 1 shows a significant increase in FIAF after co-incubation of Caco-2 (secreted fraction), and HT-29 (soluble fraction) cells with *E. faecalis* LMG 7937, whereas secretion levels were lowered in HCT-116 cells. *E. coli* significantly decreased FIAF secretion in HCT-116 cells, whereas *B. thetaiotaomicron* significantly increased secreted levels by the same cells.

Bacterial adhesion on Caco-2 cells and metabolite production

To investigate whether bacterial adhesion was involved in the observed FIAF effects, we determined the amount of adhered bacteria to the Caco-2 cells by flow cytometry (Table 2A). However, no correlation could be found between the amount of adhered bacteria and the effects on FIAF isoform type or expression. In a next step, we tested whether specific metabolite production by the bac-

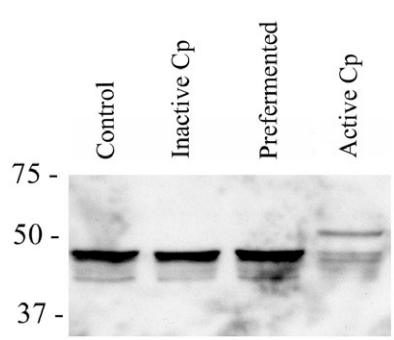

Fig. 2. FIAF isoform change for differentiated Caco-2 cells treated with *C. perfringens*. Western blot data for differentiated Caco-2 cells incubated for 2 hours with DMEM (Control), DMEM with heat-treated *C. perfringens* (Inactive Cp), cell free DMEM prefermented with *C. perfringens* (Prefermented) and DMEM with live *C. perfringens* (Active Cp).

Table 1. FIAF isoform change and average relative FIAF intensity (± SEM) based on the FIAF band intensities in the different protein fractions (insoluble: membrane/nucleus, soluble: cytoplasm, secreted) of Caco-2, HT-29 and HCT-116 cells co-incubated with different intestinal bacteria compared with untreated cells.

Cell line	Species	Isoform change	Insoluble (50 kDa)	Soluble (41–45 kDa)	Soluble (50 kDa)	Secreted (67 kDa)	Secreted (32 kDa)
Caco-2	*B. thetaiotaomicron*	No	0.9 ± 0.19	0.8 ± 0.27	n.d.	1.4 ± 0.94	n.d.
	E. coli	No	1.1 ± 0.44	0.8 ± 0.38	n.d.	1.1 ± 0.46	n.d.
	B. breve	No	1.8 ± 1.48	0.9 ± 0.19	n.d.	1.7 ± 0.98	n.d.
	L. brevis	No	0.9 ± 0.19	1.2 ± 0.34	n.d.	1.8 ± 4.14	n.d.
	C. perfringens	Yes	1.0 ± 0.19	0.4 ± 0.09*	1.6 ± 0.17**	0.8 ± 0.35	n.d.
	E. faecalis LMG 7937	No	0.8 ± 0.38	1.4 ± 1.92	n.d.	2.7 ± 1.25**	n.d.
HT-29	*B. thetaiotaomicron*	No	0.8 ± 0.06	1.2 ± 0.11	n.d.	1.1 ± 0.06	n.d.
	E. coli	No	0.8 ± 0.23	0.7 ± 0.24	n.d.	0.8 ± 0.19	n.d.
	B. breve	No	1.2 ± 0.36	1.0 ± 0.11	n.d.	1.0 ± 0.18	n.d.
	L. brevis	No	0.9 ± 0.18	1.1 ± 0.04	n.d.	1.2 ± 0.24	n.d.
	C. perfringens	No	0.9 ± 0.17	0.8 ± 0.15	n.d.	0.9 ± 0.11	n.d.
	E. faecalis LMG 7937	No	0.7 ± 0.26	1.9 ± 0.53**	n.d.	0.9 ± 0.14	n.d.
HCT-116	*B. thetaiotaomicron*	No	1.0 ± 0.13	0.9 ± 0.15	n.d.	1.2 ± 0.29	1.7 ± 0.35
	E. coli	No	1.0 ± 0.11	0.9 ± 0.11	n.d.	0.5 ± 0.18*	0.5 ± 0.40*
	B. breve	No	1.1 ± 0.05	0.8 ± 0.10	n.d.	1.0 ± 0.39	1.2 ± 0.19
	L. brevis	No	1.7 ± 0.70	1.2 ± 0.17	n.d.	1.0 ± 0.30	0.9 ± 0.20
	C. perfringens	No	0.9 ± 0.12	0.9 ± 0.11	n.d.	1.0 ± 0.29	1.4 ± 0.45
	E. faecalis LMG 7937	No	0.8 ± 0.10	0.9 ± 0.10	n.d.	0.7 ± 0.12*	1.2 ± 0.20

Grey boxes highlight the results that are significantly different. Single asterisk (*) and double asterisk (**) indicate values that are significantly lower/higher than the control condition respectively (double-sided *t*-test, $3 \leq n \leq 6$, $P < 0.05$). n.d., not detected.

teria might be responsible for the observed effects. When measuring the SCFA content in culture medium after incubation with bacteria alone, none of the tested SCFAs was detected, except for acetate, which was always lower than 1.3 mM. Furthermore, the H_2O_2 concentration was always lower than 0.01 mM (Table 2A). Hence, no correlation was found between the tested metabolites production or pH and FIAF isoform or expression modulation.

SCFAs stimulate FIAF expression

Because a 2 h incubation with bacteria was too short to measure detectable metabolite levels, and because it was reported earlier that SCFA and H_2O_2 modulate the expression and activity of transcription factors PPARγ and Hif-1α, we decided to incubate Caco-2, HT-29, HCT-116 and HepG2 cells with the pure microbial metabolites, and this for longer incubation times. We included the HepG2 cells since SCFAs are circulating microbial metabolites that can be absorbed by the liver. Table 3 gives an overview of the changes in FIAF isoforms and expression levels in the different protein fractions obtained from Caco-2, HT-29, HCT-116 and HepG2 cells after 24 h of incubation with acetate, propionate and butyrate.

In summary, two overall conclusions could be made from Table 3. First, no effects on FIAF isoform type or expression could be observed when the different cell lines were treated with acetate. Second, both propionate and

Table 2. Total amount of bacteria in the medium and of bacteria adhered to the Caco-2 cells after 2 h of incubation, and their viability (Viab, average ± STD, $n = 3$).

Bacterium	$Total_{added}$ (log)	$Viab_{added}$ (%)	$Total_{adhered}$ (log)	$Viab_{adhered}$ (%)	Acetate (mM)	ΔpH	H_2O_2 (μM)
A.							
B. breve	8.4 ± 0.07	42 ± 7.9	7.0 ± 0.16	89 ± 2.5	0.3 ± 0.05	0.1 ± 0.02	1.2 ± 8.00
B. thetaiotaomicron	8.9 ± 0.03	94 ± 2.6	8.1 ± 0.04	92 ± 1.5	1.1 ± 0.06	–0.5 ± 0.05	3.2 ± 0.03
C. perfringens	8.7 ± 0.06	95 ± 2.2	7.6 ± 0.20	91 ± 0.8	0.6 ± 0.05	–0.3 ± 0.03	1.5 ± 3.27
E. coli	8.7 ± 0.06	99 ± 0.5	7.6 ± 0.06	95 ± 0.3	1.2 ± 0.07	–0.3 ± 0.03	0.3 ± 1.77
E. faecalis LMG 7937	9.0 ± 0.05	99 ± 0.1	7.3 ± 0.05	96 ± 0.2	0.3 ± 0.03	–0.1 ± 0.01	0.0 ± 1.78
L. brevis	9.5 ± 0.01	98 ± 1.7	8.1 ± 0.25	98 ± 5.5	0.3 ± 0.02	0.0 ± 0.01	0.0 ± 1.62
B.							
E. faecalis (OG1RF)	9.1 ± 0.11	97 ± 0.3	7.5 ± 0.27	89 ± 7.2	1.0 ± 0.2	n.d.	101.7 ± 5.97
E. faecalis (WY84)	9.0 ± 0.01	97 ± 2.0	7.5 ± 0.35	90 ± 6.2	0.7 ± 0.06	n.d.	16.9 ± 0.50

The viability is expressed as the percentage of living bacteria compared with the sum of living, intermediate and dead bacteria. The difference in pH and acetate and H_2O_2 concentrations were measured after 2 h incubation of DMEM with bacteria, without Caco-2 cells ($n = 3$). No production of propionate, butyrate, isobutyrate, valerate, isovalerate, caproate or isocaproate could be detected.

Table 3. FIAF isoform change and average relative FIAF intensity (± SEM) based on the FIAF band intensities in the different protein fractions (insoluble: membrane/nucleus, soluble: cytoplasm, secreted) of Caco-2, HCT-116, HT-29 and HepG2 cells co-incubated with 10 mM acetate, propionate and butyrate for 24 h compared with untreated cells.

		FIAF cleavage	Insoluble (50 kDa)	Soluble (41–45 kDa)	Secreted (67 kDa)	Secreted (32 kDa)
Caco-2	Acetate	No	0.9 ± 0.32	n.d.	1.0 ± 0.12	1.3 ± 0.22
	Propionate	Yes	0.8 ± 0.12	n.d.	2.1 ± 0.36**	1.7 ± 0.04**
	Butyrate	Yes	0.7 ± 0.30	n.d.	1.2 ± 0.07**	2.7 ± 0.65**
HT-29	Acetate	No	1.4 ± 0.32	1.0 ± 0.03	0.9 ± 0.01	1.4 ± 0.18
	Propionate	Yes	10.1 ± 0.84**	0.7 ± 0.09*	0.9 ± 0.04	2.6 ± 0.43**
	Butyrate	Yes	17.7 ± 2.75**	0.3 ± 0.07*	1.0 ± 0.04	1.7 ± 0.11**
HCT-116	Acetate	No	1.3 ± 0.16	1.3 ± 0.68	1.0 ± 0.11	1.0 ± 0.62
	Propionate	Yes	1.3 ± 0.33	1.0 ± 0.22	1.0 ± 0.21	2.9 ± 2.23**
	Butyrate	No	1.2 ± 0.30	0.7 ± 0.03*	0.4 ± 0.05*	1.3 ± 0.48
HepG2	Acetate	No	0.9 ± 0.27	1.3 ± 0.30	1.0 ± 0.24	1.0 ± 0.24
	Propionate	No	3.3 ± 0.39**	0.8 ± 0.10*	0.7 ± 0.06*	1.1 ± 0.39
	Butyrate	Yes	5.3 ± 3.64**	0.8 ± 0.10*	0.5 ± 0.01*	1.9 ± 0.4**

Results with a grey background indicate significant differences. Single asterisk (*) and double asterisk (**) indicate values that are significantly lower and higher than the control condition respectively (based on double-sided *t*-test, $n = 3$, $P < 0.05$).

butyrate are able to modulate secretion of one or both FIAF isoforms not only in the colon-derived cell lines but also in the hepatic HepG2 cells. A representative immunoblot is presented in Fig. 3A showing an appearance of the 32 kDa FIAF isoform in the secreted protein fraction of Caco-2 cells after treatment with propionate and butyrate (10 mM), but not with acetate. Figure 3B shows the overall secreted protein fraction after treatment of Caco-2 cells with SCFAs for 24 h. Apparently, FIAF is secreted in a non-specific way by high concentrations of propionate and butyrate, since secretion of other proteins seem were generally increased as well. Since we hypothesized that this stimulated secretion is the result of increased transcription and translation of FIAF, we checked the FIAF expression levels in the insoluble and soluble fractions. We found a correlation between increased FIAF secretion and higher levels in the insoluble fraction of HT-29 and HepG2 cells. In these cell lines, PPARγ was significantly increased in the insoluble protein fraction (3.9- and 3.5-fold in HepG2 for propionate and butyrate, respectively, and 1.3-fold in HT-29 cells for propionate). In the Caco-2 cells, a tendency towards an increased PPARγ expression could be noticed within 4 h of incubation (data not shown). PARP cleavage was observed when HepG2 cells were incubated with butyrate (10 mM), which indicates apoptosis under these conditions, whereas the other cell lines were unaffected.

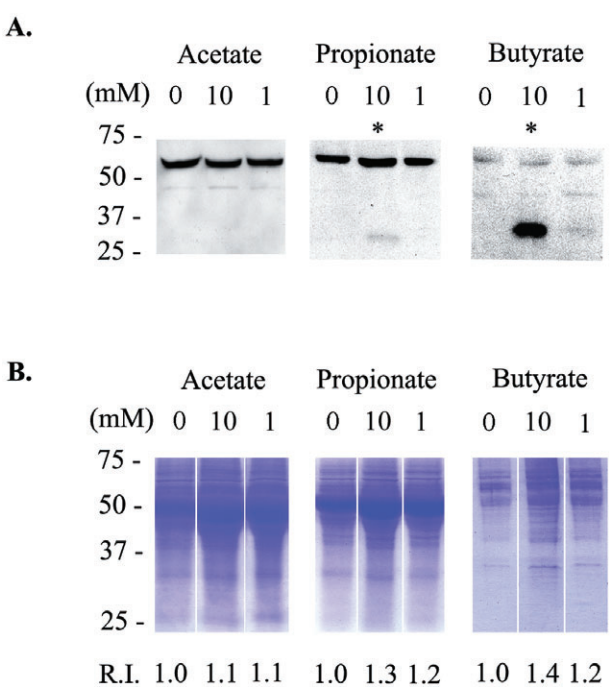

Fig. 3. FIAF isoform and concentration change for Caco-2 cells treated with acetate, propionate and butyrate. (A) Western blot detection of different secreted FIAF products from differentiated Caco-2 cells treated with SCFA after 24 h. (B) Coomassie blue staining of the gel showing the total amount of proteins secreted by differentiated Caco-2 cells treated with acetate, propionate and butyrate in different concentrations (0, 10 and 1 mM) after 24 hours. R.I., relative intensity *: indicate values which are significantly higher that the control condition, respectively.

H_2O_2 and H_2O_2-producing E. faecalis *strains modulate FIAF expression, secretion and isoform change*

H_2O_2 is a microbial metabolite that also has an influence on the transcription factors PPARγ and Hif-1α. Therefore, the effect of H_2O_2, the strong H_2O_2-producing *E. faecalis* mutant strain OG1RF, and the mild H_2O_2-producing *E. faecalis* WY84 wild-type strain on FIAF isoform changes and concentrations by differentiated Caco-2 cells was investigated (Table 4). A first observation was that incubation with H_2O_2 resulted in a significant effect on the different FIAF isoforms in both the soluble and secreted

Table 4. FIAF isoform change and average relative FIAF intensity (± SEM) based on the FIAF band intensities in the different protein fractions (insoluble: membrane/nucleus, soluble: cytoplasma, secreted) of differentiated Caco-2 cells co-incubated with H_2O_2, *E. faecalis* OG1RF (mutant) and *E. faecalis* WY84 (wild type) compared with untreated Caco-2 cells.

Differentiated Caco-2 2 h incubation	Insoluble	Soluble		Secreted	
FIAF isoform change					
H_2O_2	No	Yes: ≥ 0.01 mM		Yes: ≥ 1 mM	
E. faecalis OG1RF	No	Yes		No	
E. faecalis WY84	No	Yes		No	
	Insoluble (50 kDa)	Soluble (45–41 kDa)	Soluble (50 kDa)	Secreted (67 kDa)	Secreted (32 kDa)
FIAF concentration					
H_2O_2 (1 mM)	1.1 ± 0.03	0.6 ± 0.20*	1.5 ± 0.28**	1.0 ± 0.10	1.3 ± 0.15**
E. faecalis OG1RF	1.0 ± 0.00	0.5 ± 0.03*	6.0 ± 1.34**	1.3 ± 0.03**	n.d.
E. faecalis WY84	1.1 ± 0.05	0.7 ± 0.31	4.5 ± 1.12**	1.3 ± 0.07**	n.d.

Results with a grey background indicate significant differences and single asterisk (*) and double asterisk (**) indicate values that are significantly lower and higher than the control condition respectively (based on double-sided *t*-test, $n = 3$, $P < 0.05$).

protein fraction (Fig. 4). In analogy with when Caco-2 cells were treated with *C. perfringens*, the 41–45 kDa FIAF was significantly lowered in the soluble fraction and simultaneously a new band with a MM of 50 kDa appeared (Fig. 4A). A 2 h incubation of Caco-2 cells with different concentrations of H_2O_2 (0, 0.001, 0.01, 0.1 and 1 mM) showed that the FIAF isoform change in the soluble protein fractions was visible at concentrations above 0.01 mM (data not shown). A kinetic experiment with 0.1 mM H_2O_2 showed that the 50 kDa FIAF isoform change already appeared within 30 min of treatment (data not shown). As observed with butyrate and propionate, a cleavage of the secreted FIAF of approximately 67 kDa was visible upon treatment with H_2O_2 at concentrations above 1 mM, resulting in the appearance of a band of approximately 32 kDa (probably the N-glycosylated 26 kDa form) (Fig. 4B).

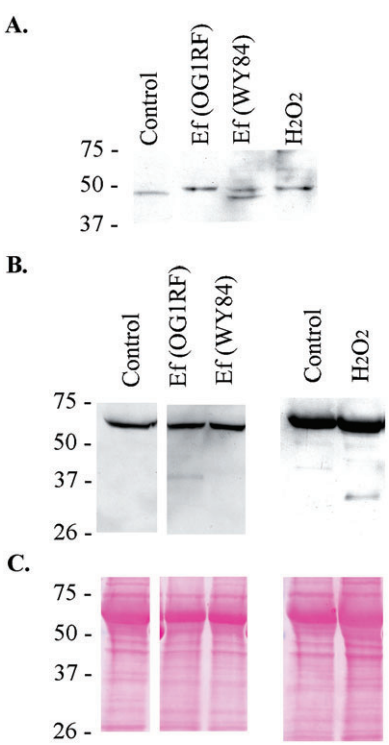

Fig. 4. FIAF isoform changes for Caco-2 cells treated with H_2O_2 (1mM) and H_2O_2 producing *E. faecalis* strains. Western blot results for the soluble (A) and secreted protein fraction (B) of differentiated Caco-2 cells. Ef(OG1RF) is the strong H_2O_2 producing mutant strain of *E. faecalis*, whereas Ef(WY84) is the mild H_2O_2 producing wild type strain. (C) Ponceau red staining of the blot as loading control of the total amount of secreted proteins.

Analysis of the H_2O_2 production from the *E. faecalis* OG1RF mutant and its wild-type strain *E. faecalis* WY84 (Table 2B) showed that the mutant strain produced much more H_2O_2 (0.102 mM) than the wild type after 2 h, although a significant H_2O_2 production was also noted for the latter (0.017 mM). This explains why both the mutant and wild-type strain induced a similar FIAF isoform change in the soluble protein fraction when co-incubated with differentiated Caco-2 cells as did H_2O_2 (Table 4). The H_2O_2 production by both strains was however too low to induce cleavage of the 67 kDa FIAF to a 32 kDa isoform in the secreted FIAF fraction. Interestingly, the secreted 67 kDa FIAF levels were significantly increased with both *E. faecalis* OG1RF and *E. faecalis* WY84 (Table 4 and Fig. 4), which was in parallel with the results for *E. faecalis* LMG 7937. No significant changes in PPARγ, Hif-1α, LDH, PARP or LC3 were observed in this experiment.

Discussion

In this study, we found that certain members of the gut microbiota, such as *E. faecalis*, *C. perfringens*, *B. thetaiotaomicron* and *E. coli* alter FIAF production in colorectal cancer cell lines. Furthermore, SCFAs, such as butyrate

and propionate, and H_2O_2, were identified for the first time as microbial factors that actively modulate FIAF production and/or isoform structure. Because different FIAF isoforms have different physiological effects, this article provides some mechanistic clues about the differential impact of gut microbiota on various host health beneficial effects through FIAF modulation.

In vivo studies on microbial regulation of FIAF are often conflicting. Microbial regulation of intestinal FIAF was first reported by Bäckhed and colleagues (2004), who found that gnotobiotic mice, conventionalized with an intestinal microbial community, resulted in decreased intestinal FIAF expression. Because FIAF inhibits LPL activity, it was hypothesized that the decrease in circulating FIAF was responsible for the increased fat storage in the adipose tissue. FIAF repression in the intestine upon conventionalization was also demonstrated in a similar study by Fleissner and colleagues (2010). However, this research group also reported that, unexpectedly, plasma FIAF levels were not decreased in conventionalized compared with germfree mice. Moreover, in conventionalized mice, a higher concentration of cleaved FIAF was observed whereas the native FIAF concentration was unchanged. These results suggest that (i) the intestine may not be the major source of FIAF in the body, (ii) that the microbial community may increase FIAF in sites of the body other than the intestine, and (iii) the FIAF isoform (native versus cleaved) may be of importance for the physiological effects of FIAF. In addition, recent publications suggest that the FIAF repressing potential of a microbial community may depend on the FIAF regulating potential of the individual members of this community, and to the metabolites produced by this community (Aronsson *et al*., 2010). Indeed, Bäckhed and colleagues (2007) reported that the combination of *B. thetaiotaomicron* and *Methanobrevibacter smithii* resulted in both a more efficient saccharolytic fermentation and a stronger repression of FIAF in mice conventionalized with these bacteria. In addition, Aronsson and colleagues (2010) reported that FIAF was upregulated in colon cancer cells by a secretion product of *Lactobacillus paracasei* ssp. *paracasei* F19. The secretion products, however, were not identified in these studies, and hence, the mechanisms of FIAF regulation in both the intestine and the entire body remain unclear.

In this study, we therefore chose an *in vitro* approach using different colorectal cancer cell lines to gain more insight in the mechanisms by which gut bacteria modulate (i) FIAF expression and (ii) FIAF isoform type. This allowed us to screen for several microbial parameters in a controlled cell environment. In addition, we used Western blot as a detection method for the different FIAF isoforms, because other techniques such as ELISA and qPCR are not able to distinguish between the different FIAF isoforms. Three colorectal cancer cell lines were selected according to the following criteria: (i) the differentiation level, in order to compare a differentiated enterocyte phenotype with a cancer cell phenotype, because FIAF has been shown to be upregulated during differentiation (Yoon *et al*., 2000; Dutton and Trayhurn, 2008), and (ii) TLR4 expression, because LPS from Gram-negative bacteria has been shown to upregulate FIAF through a TLR4 mediated mechanism (Brown *et al*., 2009; Lu *et al*., 2010). With respect to these criteria, the Caco-2 cell line was chosen as a model for differentiated intestinal epithelium, because these cells are able to spontaneously differentiate into enterocyte-like cells upon confluency (Chantret *et al*., 1988). In addition, since these cells do not express TLR4, they are suitable to investigate LPS-independent processes (Takahashi *et al*., 2009), in contrast to the HT-29 cell line that was chosen to study TLR4-mediated processes (Zhao *et al*., 2007). Because TLR4 expression in HT-29 cells is downregulated upon differentiation (Kim *et al*., 2008), we prevented differentiation by culturing the cells in glucose-rich medium (Zweibaum *et al*., 1985). Finally, the HCT-116 cell line was selected because it is a highly aggressive cell line that shows a low ability to differentiate and because its TLR4 expression is also low (Ishizu *et al*., 2007; Rajput *et al*., 2008). Therefore the results with this cell line may be relevant in the context of microbial modulation of colon cancer progression. We found that the low metastatic Caco-2 and HT-29 cell line only secreted detectable amounts of the 67 kDa FIAF isoform, whereas the strong metastatic and invasive HCT-116 and HepG2 cell line (Yu *et al*., 2010) secreted both the native 67 kDa and cleaved 32 kDa FIAF isoforms. Until now, the function of the cleaved FIAF isoform in cancer progression was never investigated. Hence, these data suggest that the microbial regulation of FIAF in these cell lines should be carefully interpreted in the context of obesity, because also cancer-related physiological processes may be involved.

LPS is a circulating factor of microbial origin that has been reported to increase FIAF production in neuronal and adipocyte cells (Brown *et al*., 2009; Lu *et al*., 2010). However, our work shows that it is unlikely that microbial LPS is responsible for the FIAF modulation. First, LPS-negative bacteria like *E. faecalis* and *C. perfringens* were able to influence FIAF expression and secretion. Second, FIAF levels were not modulated by LPS-releasing Gram-negative bacteria, such as *B. thetaiotaomicron* and *E. coli* in a TLR4-positive cell line. In contrast, we found increased levels of the secreted FIAF cleavage product in the HCT-116 cell line after treatment with this bacterium. Moreover, conventionalization of mice with a monoculture of *B. thetaiotaomicron* has previously been shown to decrease intestinal FIAF expres-

sion (Bäckhed *et al*., 2007). Therefore, the effect of LPS on FIAF expression in the colon remains unclear.

To the best of our knowledge, we identified for the first time propionate and butyrate as potential FIAF stimulating microbial metabolites not only in the three tested colonic cancer cell lines, but also in a hepatic cancer cell line. We showed that propionate and butyrate not only stimulate the secretion but also the cleavage of FIAF. SCFAs concentrations in the human gut range from 131 mmol kg^{-1} in the caecum to 80 mmol kg^{-1} in the descending colon at an acetate : propionate : butyrate ratio of roughly 60:20:20 (Cummings, 1981; Cummings *et al*., 1987). A substantial amount of SCFA is absorbed in the bloodstream, leading to SCFA concentration of 375 μM (69:23:8), 148 μM (78:14:8) and 79 μM (89:6:5) in the portal, hepatic and peripheral bloodstream respectively (Cummings *et al*., 1987). Therefore, circulating propionate and butyrate may explain the increase in cleaved FIAF in blood plasma of conventionalized mice reported by Fleissner and colleagues (2010).

What are then the microbial factors responsible for FIAF repression in the intestine? From our results, we identified only one strain, namely *E. coli* that strongly reduced FIAF secretion. However, this was restricted to the highly aggressive and non-differentiated HCT-116 cells. A similar cell line-dependent response was reported by Bhattacharjee and colleagues (2005) who showed that *E. coli*-derived verotoxin 1 induced apoptosis in HCT-116 cells but not in Caco-2 and HT-29 cells. However, no verotoxin production was detected in the tested *E. coli* strain (data not shown). *Clostridium perfringens* is a toxin-producing bacterial strain that decreased the 45–41 kDa FIAF isoforms in parallel with a 50 kDa isoform increase. A similar FIAF isoform change was visible in Caco-2 cells upon treatment with H_2O_2 and H_2O_2-producing *E. faecalis* strains. Therefore, it may be possible that a harmful intestinal environment rich in toxins and H_2O_2 is responsible for epithelial FIAF repression.

Apart from FIAF repression in the intestine, there is evidence that bacteria also can stimulate FIAF expression. In a very recent study from Aronsson and colleagues (2010), it was shown that *L. paracasei* ssp. *paracasei* F19, *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis BB12*, and not *B. thetaiotaomicron*, were able to stimulate FIAF mRNA expression in several colonic cell lines including HCT-116, HT-29, LoVo and SW480 cells. This effect was shown to be PPARα and PPARγ dependent. For *Lactobacillus* F19, the FIAF stimulatory effect was attributed to a secreted microbial factor, which was however not identified in the study. In addition, conventionalization of mice with *Lactobacillus* F19 increased native FIAF levels in blood plasma, and resulted in decreased fat storage and increased blood very low-density lipoprotein (VLDL) levels. In our study, we found a tendency towards higher FIAF expression with *L. brevis* in Caco-2 and HCT-116 cells with and *B. breve* in Caco-2 cells. These results were highly variable and were therefore not significant, which may be due to the different FIAF detection method (Western blot versus qPCR) and the limited incubation (2 h versus 6 h). Increased FIAF levels in native FIAF were also observed when Caco-2 and HT-29 cells were treated with *E. faecalis* strains. This process was independent of H_2O_2 production. In previous studies, incubation of colonic cell lines with *E. faecalis,* isolated from newborn babies, resulted in phosphorylation of PPARγ and increased transcription of FIAF (Are *et al*., 2008). Therefore, it is possible that also in our study FIAF is regulated via PPARγ activation through phosphorylation. Yet, the microbial factor produced by *E. faecalis* responsible for FIAF stimulation, and the possible health implications, remain to be elucidated.

To conclude, with our cell line models, we were able to identify some microbial parameters that may explain the conflicting *in vivo* results from other studies. We found that (i) in colon cancer cell lines, FIAF regulation in terms of expression levels and isoform appearance was bacterial species and cell type specific, and (ii) SCFAs as secreted microbial metabolites are able to upregulate FIAF in all tested colon and hepatic cancer cell lines. The latter suggests that (i) their FIAF-modulating effect may not be restricted to the colon epithelium itself and (ii) especially the cleaved FIAF isoform is involved in the microbial regulation of fat storage through FIAF. The outcome of these screening experiments needs further confirmation in more complex models of the host and intestinal microbial communities. From a nutritional point of view, it would be interesting to examine whether modification of the composition of the intestinal microbial community by pro- and/or prebiotics might affect FIAF production and isoform appearance and thus overall health status. Our current research is therefore focused on the functional and mechanistic *in vivo* implications of secreted FIAF on a variety of tissues and on other modulators of FIAF release in order to unravel their potential health beneficial effects.

Experimental procedures

Cell culture

Caco-2 (ATCC HTB37), HT-29 (ATCC HTB38), HCT-116 (ATCC CCL247) and HepG2 (ATCC HB-8065) cells were cultivated in DMEM supplemented with Glutamax (Gibco, Langley, USA) for Caco-2 cells, and DMEM (Gibco) for the other cell lines. The media were supplemented with 10% fetal bovine serum (FBS, Greiner Bio-One, Wemmel, Belgium), 1% non-essential amino acids, 2% penicillin/streptomycin and transferrin (Invitrogen, Merelbeke, Belgium) at 10% CO_2 in air. Caco-2 cells were grown to 95% confluency in T75

culture flasks to obtain undifferentiated cells, whereas differentiated Caco-2 cells were obtained by culturing them for 21 days. The other cell lines were cultured for 14 days. For the transwell experiments, Caco-2 cells were seeded on Transwell® plates (0.4 μm pore diameter, 6.5 mm diameter, Corning Costar, New York, USA) at a density of 25 000 cells per well and incubated for 21 days to allow differentiation. Cell toxicity was assessed by measuring lactate dehydrogenase (LDH) release in the culture medium using the Cytotoxicity Detection Kit Plus (Roche, Vilvoorde, Belgium).

Bacterial culture

Bifidobacterium breve, *B. thetaiotaomicron* (ATCC 29148), *E. faecalis* (LMG 7937), *E. faecalis* OG1RF (Huycke et al., 2002), *E. faecalis* WY84 (Huycke et al., 2002), *C. perfringens* (LMG 11264), *E. coli* (CM 120) and *L. brevis* (LMG 120123) were cultivated overnight in Brain Heart Infusion broth (Gibco, Invitrogen, Merelbeke, Belgium), at 37°C and under anaerobic conditions. The *B. breve* strain was previously isolated from the Yakult Bifiel product and its species identity was confirmed by BOX-PCR fingerprinting (Masco et al., 2005).

Treatment of colorectal cancer cells with live intestinal bacteria

The bacterial cultures were washed trice with saline solution (8.5 g l^{-1} NaCl, centrifugation during 5 min, 4000 *rcf*) and suspended in an equal volume of serum and antibiotic-free DMEM. The optical density of 100 μl of sample, measured at 620 nm with a Sunrise multiwell reader (Tecan, Männedorf, Switzerland), was adapted to 0.5. This corresponded to a total amount of 8.4–9.5 log bacteria per condition, as determined by flow cytometry (see further), and is consistent with the amount of bacteria used in similar co-incubation experiments (Henriksson *et al.*, 1991). Immediately after, colorectal cancer cells were washed trice with phosphate-buffered saline solution and co-incubated with 10 ml of this bacteria-containing medium during 2 h at 37°C and in presence of 10% CO_2. In case of the transwell experiments, 200 μl of bacterial suspension was added to the apical side of the Caco-2 cell monolayer while 1 ml of sterile medium was added to the basal side.

Caco-2 cells treated with live and heat-inactivated C. perfringens *and pre-fermented DMEM*

To obtain dead bacterial cells, approximately 9 log bacteria were washed with saline solution. The pellet was incubated in a hot water bath at 70°C for 1 h, and resuspended in serum and antibiotic-free DMEM. To obtain pre-fermented DMEM, an equal amount of living bacteria were suspended in DMEM and incubated at 37°C for 2 h, without being in contact with Caco-2 cells. Thereafter, the suspension was centrifuged (5 min, 4000 *rcf*) and the supernatant was sterilized with a 0.22 μm filter (Millipore, Billerica, USA) and retained as the pre-fermented medium. Serum and antibiotic-free DMEM, heat-inactivated bacteria, pre-fermented DMEM and living bacteria were added to the Caco-2 cells and incubated for 2 h at 37°C and in presence of 10% CO_2.

Treatment of colorectal and hepatic cancer cells with bacterial metabolites

H_2O_2, sodium acetate, sodium propionate and sodium butyrate were purchased from Sigma-Aldrich Corporation (Saint Louis, USA). SCFAs were added at a concentration of 1 and/or 10 mM. H_2O_2 was added at a concentration of 0.001, 0.01, 0.1 and 1 mM.

Preparation of protein fractions

Secreted protein fraction. Upon treatment, the culture medium of the T75 culture flask or apical and basal fraction of the Transwell plates was centrifuged (3000 *rcf*, 10 min), and the supernatant was transferred through a 0.22 μm sterile filter (Millipore). The medium was then concentrated by ultracentrifugation (10 kDa filter; Millipore, 40 min, 4000 *rcf*), until ±250 μl of retentate was obtained. Small variations in end volume were adjusted to an equal volume by adding sterile PBS. Peptide-*N*-glycosidase F (PNGase F, Roche, Mannheim, Germany) was used to check N-glycosylation of the secreted protein fraction (Yang *et al.*, 2008).

Soluble protein fraction. The Caco-2 cells were washed and treated with 1 ml of mild lysis buffer, containing 1% Triton X-100 and 1% NP-40 in Ca^{2+}/Mg^{2+}-supplemented PBS, and protease inhibitors (2 M PMSF, 10 mg l^{-1} leupeptin, 10 mg l^{-1} apoprotinin, 1 mM $NaVO_3$, 2.5 g l^{-1} $Na_4P_2O_7$ and 0.1 mM NaF). The lysate was centrifuged (10 min, 12 000 *rcf*). The supernatant is referred to as the 'soluble' protein fraction in the text.

Insoluble protein fraction. The pellet of the above described procedure was dissolved in 250 μl of Laemmli 1× lysis solution (0.125 M Tris-HCl, 123 g l^{-1} glycerol), sonicated during 10 s and centrifuged (2000 *rcf*, 10 min). The supernatant is referred to as the 'insoluble' fraction in the text.

Western blot and immunostaining

Protein concentrations were determined with the Protein Assay kit (Bio-Rad, Hercules, USA). In case of the insoluble and soluble fraction, an equal amount of proteins was loaded on the polyacrylamide gel (10%), whereas an equal volume of the sample was loaded in case of the secreted protein fraction. As extra loading control for the secreted protein fraction, a Ponceau Red staining of the blot or a Coomassie blue staining of the gel was scanned. After blotting, the proteins of interest were visualized by immunostaining. Polyclonal primary antibodies used in the study were the anti-FIAF (Atlas Antibodies, Stockholm, Sweden), anti-PPARγ (Biovendor, Modrice, Czech Republic) and anti-Hif-1α (Upstate, Billerica, USA) antibodies. Monoclonal anti-tubulin (Sigma) and polyclonal anti-H2A antibodies (Upstate) were used as a loading control in the soluble and insoluble protein fraction respectively. Cell apoptosis and autophagy were

checked by immunostaining of cleavage products of poly-(ADP-ribose) polymerase (PARP) (mouse monoclonal antibody, BD Biosciences, San Jose, USA) and microtubule-associated protein 1 light chain 3 (LC3) (rabbit monoclonal antibody, Sigma) respectively. The peroxidase labelled anti-rabbit and anti-mouse secondary antibodies were purchased from GE Healthcare (Buckinghamshire, UK). Bands were visualized by autoradiography, using ECL reagent (Pierce, Rockford, USA), photo-sensitive films (Amersham, GE Healthcare) and a Kodak film developer (Eastman Kodak Company, Rochester, USA).

Flow cytometric live/dead detection of bacteria

To determine the amount and viability of adhered bacteria, a live/dead staining of the bacteria was performed and detected by flow cytometry (Cyan ADP, Dako, Glostrup, Denmark) with the Summit v3.4 software. The flow cytometric protocol was optimized previously. Briefly, equal samples of the Caco-2 cell monolayer were treated with 0.2% Triton X-100 in filter-sterilized drinking water during 10 min, and vigorously vortexed to detach the bacteria from the eukaryotic cells. After centrifugation (5 min, 3000 *rcf*), the pellet was resuspended in filter-sterilized water. Staining was performed using SYBR Green and propidium iodide (Live/dead BacLight Bacterial Viability kit, Invitrogen, Merelbeke, Belgium).

SCFA, H_2O_2 and pH analysis

Short-chain fatty acid (acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate and isocaproate) concentrations were determined using a diethyl ether extraction as described before (De Boever *et al*., 2000). A GC-2014 (Shimadzu) with FID detector and split injector was used. The injection volume was 1 μl and the temperature profile was set from 110°C to 160°C, with a temperature increase of 6°C min^{-1}. The column was a capillary fatty acid-free column (EC-1000 Econo-Cap column, Alltech, Laarne, Belgium, 25 m × 0.53 mm; film thickness 1.2 μm). The carrier gas was nitrogen, and the temperature of the injector and detector were 200°C and 220°C respectively. H_2O_2 analysis was performed on 10 μl of sample in 190 μl of FOX-1 reagent (Banerjee *et al*., 2004) and detected at 560 nm with a Paradigm spectrophotometer (Beckman Coulter, Woerden, the Netherlands). The pH was measured with a Consort SP10B electrode connected to a Consort C532 multimeter analyser (Turnhout, Belgium).

Statistics

Quantification of the bands was performed with the program Quantity one 4.6.6. (Bio-Rad). A two-way Student's *t*-test was performed to check significance ($P < 0.05$).

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