British Journal of Nutrition (2012), **108**, 2043–2053
doi:10.1017/S0007114512000220

In vitro comparison of commensal, probiotic and pathogenic strains of *Enterococcus faecalis*

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(Submitted 7 September 2011 – Final revision received 18 November 2011 – Accepted 3 January 2012 – First published online 21 February 2012)

Abstract

In vivo studies have provided evidence that micro-organisms have important roles in immunological, digestive and respiratory functions, conferring health benefits on the host. Several *in vitro* methods have been advised for the initial screening of microbes with potential health effects. The objective of the present study was to employ such *in vitro* methodology to characterise different strains of *Enterococcus faecalis*. The characteristics of a commercial product marketed as a probiotic, Symbioflor-1 (Symbiopharm), were compared with the characteristics of both pathogenic and commensal strains. Tolerance towards low pH and viability after exposure to human gastric and duodenal juices were assayed. Symbioflor-1 was the most susceptible strain to these treatments when compared with the other *E. faecalis* strains. Furthermore, Symbioflor-1 exhibited the lowest adhesion capacity to intestinal epithelial cells (IEC) and mucus. Competitive binding studies using heparin indicated that glycosaminoglycans might be involved in the adhesion to IEC, but also that differences in these putative bacteria–host interactions do not cause the relative low adhesion capacity of Symbioflor-1. Maturation of dendritic cells (DC) after exposure to bacteria was assayed as an indication of an immunomodulatory effect. All strains induced a moderate elevation of the DC maturation markers CD83 and CD86; however, no strain-specific differences were detected. Correlations between *in vitro* and *in vivo* studies are discussed. Although *in vitro* assaying is a rational starting point for the selection of microbes with a potential health benefit, it is emphasised that human clinical trials are the definite tool for establishing probiotic status.

Key words: Probiotics: *Enterococcus faecalis*: Symbioflor-1

Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'[1]. Lactic acid bacteria and bifidobacteria are commonly found in the human gastrointestinal tract (GIT), and represent the majority of commercial probiotics. Antimicrobial activity, exclusion or inhibition of pathogens, enhancement of the intestinal epithelial barrier function[2], ability to degrade indigestible food components[3] and immunomodulation[4–7] are some of the numerous beneficial effects reported. Probiotics are thereby widely used as food supplements, which calls for a proper evaluation of the microbes in order to substantiate the health claims. Guidelines for such evaluation have been provided by the FAO/WHO, with the intension to establish a set of minimum requirements for probiotic status[8]. Viability after exposure to the different challenging environments encountered in the upper GIT, ability to prolong intestinal residence by adhesion to mucus and/or intestinal epithelial cells (IEC), and the potential to modulate the immune system are commonly assayed when screening for microbes with potential probiotic properties.

The Gram-positive, facultative anaerobe lactic acid bacterium *Enterococcus faecalis* is a common component of the human commensal flora, but has received substantial attention due to its 'dualistic' behaviour toward human health[9,10]. *E. faecalis* is recognised as a multi-resistant, opportunistic pathogen and has become one of the leading causes of nosocomial infections[11,12]. The *E. faecalis* strain V583 was the first vancomycin-resistant clinical isolate reported in the USA, and represents a well-described pathogenic strain[13,14]. Intriguingly, *E. faecalis* is also made into commercial products

Abbreviations: BHI, brain–heart infusion; CFU, colony-forming units; DC, dendritic cells; GIT, gastrointestinal tract; HDJ, human duodenal juice; HGJ, human gastric juice; IEC, intestinal epithelial cells; PI, propidium iodide.

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marketed as probiotics, such as Symbioflor-1 (SymbioPharm GmbH). Symbioflor-1 has been reported to reduce the number of relapses in patients with chronic recurrent hypertrophic sinusitis, as well as the number and severity of relapses in patients with chronic recurrent bronchitis[15,16]. According to the manufacturer, these beneficial effects are due to a 'natural training effect on the immune system' initiated by bacteria–host interactions in the intestine. This immunomodulatory effect is then distributed to various mucous membranes throughout the body, and defence systems located at the paranasal sinus and bronchial tract are thereby enhanced. Beneficial effects of probiotics on respiratory tract infections (RTI) have also been reported by others[17,18]. Vouloumanou *et al.*[18] evaluated the clinical evidence regarding probiotic use for the prevention of RTI and concluded that administration of probiotics may have a beneficial effect on the severity and duration of symptoms, but does not appear to reduce the incidence of RTI.

Variations can exist in the molecular mechanisms employed by the probiotic, pathogenic and commensal *E. faecalis* strains upon host interaction. Strain-specific differences in the ability to, for example, colonise the intestine and/or elicit an immune response are likely. Such knowledge will be valuable when evaluating the risk associated with using *Enterococcus* as food starter cultures or as probiotics. As it is not possible to differentiate between virulent and a-virulent enterococcal strains, the Panel on Biological Hazards of the European Food Safety Authority (EFSA) has not included any taxonomical unit within the enterococci genus in the list of Qualified Presumption of Safety (QPS) micro-organisms[19]. Due to the increasing evidence of pathogenicity, species of *Enterococcus* will no longer be assessed unless new scientific information becomes available.

Our objective was to study different characteristics of Symbioflor-1, in comparison with the pathogenic *E. faecalis* strain V583 and three other *E. faecalis* isolates (Table 1). We used several *in vitro* methods that are commonly employed when screening for potential probiotic bacteria. In this way, our approach differs from the investigations reported where numerous microbes are assayed for putative probiotic properties.

Material and methods

Cells and culture conditions

E. faecalis strains V583[13], Symbioflor-1[20] (SymbioPharm), 62[21,22], 158B[22,23] and LMGT3208[22] were kindly provided by the Laboratory of Microbial Gene Technology and Food Microbiology (Department of Chemistry, Biotechnology and Food Science, University of Life Science, Norway). All *E. faecalis* strains were cultivated at 37°C in brain–heart infusion (BHI) medium (Oxoid). *Lactobacillus reuteri* DSM 20 016[24] and *L. plantarum* NC8[25] were kindly provided by Nofima AS and anaerobically cultivated in de Man, Rogosa and Sharpe (MRS) medium (Oxoid) at 37 and 30°C, respectively. All strains were stored at −80°C in 20 % (v/v) glycerol.

The human colon adenocarcinoma cell line Caco-2 (HTB-37™; American Type Culture Collection (ATTC)), monocytes and dendritic cells (DC) were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 2 mM-L-glutamine, 100 μM-non-essential amino acids, 1 mM-sodium pyruvate (all from PAA Laboratories), 10 % heat-inactivated fetal calf serum and gentamicin (24 mg/l) (Lonza). All experiments and maintenance of tissue culture cells were carried out at 37°C in a 5 % CO_2 humidified air atmosphere.

Assaying bacterial cultivability

Overnight cultures were diluted 1:50 in BHI medium with either neutral or HCl-adjusted pH values, as specified. Cell growth at 37°C was monitored spectrophotometrically (optical density at 600 nm) every 30 min using a Bioscreen instrument (Bioscreen C). Sterile BHI medium was used as negative control and for background correction.

Collection of physiological gastric and duodenal juices from the upper digestive tract

Human gastric juice (HGJ) and duodenal juice (HDJ) were collected from healthy volunteers. Aspiration of six fasting volunteers (aged between 20 and 30 years) was performed at Moss Hospital, Norway. The aspiration was approved by the Norwegian Ethics Committee, and written consent of participation was given. Juices were collected by placing a flexible three-lumen silicone tube (Maxter Catheters) through the nose or mouth. Correct tube placement was monitored by radiology. Instillation of a stimulatory solution (17·5 g/l sucrose, 450 mg/l NaCl, 800 mg/l L-phenylalanine and 575 mg/l L-valine in water) was performed simultaneously. Juices were collected in 50 ml tubes on ice, controlled for cross-contamination (colour and pH) and centrifuged (4500 ***g*** for 10 min) to remove mucus and cell debris. Samples were pooled, frozen as 5 ml samples and stored at −80°C. The final pH was 1·5 for the HGJ and 8·0 for the HDJ.

Table 1. *Enterococcus faecalis* strains

Strain	Country	Source	Isolation site	Characteristics	References
Symbioflor-1	Germany	Non-hospitalised individual	Faeces	Probiotic	20
V583	USA	Hospitalised patient	Blood	Vancomycin-resistant	13,14
62	Norway	Baby	Faeces	Commensal	21–23
158B	Norway	Baby	Faeces	Commensal	22,23
LMGT3208	Greece	Cheese	Cheese	Food isolate	22

Assaying cell viability by fluorescent staining and flow cytometry

Overnight bacterial cultures were diluted 1:50 in BHI medium and cultivated for 3 h at 37°C. Cells were washed and re-suspended in PBS (PAA Laboratories) to strain-specific densities (optical density at 600 nm) previously estimated to yield 1×10^8 colony-forming units (CFU)/ml by plate counting. After harvesting 0·5 ml cell suspension, the bacterial pellets were re-suspended in equal amounts of challenging or control solutions, and incubated for 30 min at 37°C. Cells were washed and re-suspended in PBS and stained with 2·5 μM-SYTO9 (Invitrogen) for 15 min at room temperature. After washing in PBS, cells were stained with 0·25 μg/ml propidium iodide (PI) (Invitrogen) for 2 min before flow cytometric analysis. SYTO9 is a membrane-permeable nucleic acid stain detected in the green channel (FL-2, fluorescein isothiocyanate), and PI is a membrane-impermeable red fluorescent nucleic acid stain detected in the red channel (FL-4, PI/phycoerythrin-Cy5-A). Fluorescence was recorded using a MACSQuant flow cytometer and data were analysed with MACSQuantify software (Milteyi Biotech). The proper staining procedure of non-viable cells was ensured by assessing heat-treated cells (70°C; 60 min). Initial cell viability was confirmed in each experiment.

Assaying bacterial adhesion to Caco-2 cells

E. faecalis strains were grown aerobically on BHI agar plates for 24 h at 37°C, and *L. plantarum* NC8 and *L. reuteri* DSM 20 016 were grown anaerobically on MRS agar plates for 48 h at 30 and 37°C, respectively. Cells were re-suspended in 5 ml PBS, washed, and re-suspended to a final concentration of 1×10^8 CFU/ml, as described above. Concentrations were verified in each experiment by plate counting. Confluent monolayers of Caco-2 cells (passage 12–25) prepared in twenty-four-well tissue culture plates (Nalge Nunc International) were washed with PBS and incubated with antibiotic-free RPMI medium for at least 1 h before infection with 2×10^6 bacteria/well. After 1 h of co-culture, non-adhered bacteria were removed by washing four times with PBS. The Caco-2 cells were lysed in 0·1 % Triton X-100 for 5 min at 37°C, and the adhered bacteria were suspended by pipetting. Adhered bacteria were diluted and plated onto appropriate agar plates by a WASP spiral plater (Don Whitley Scientific) and incubated aerobically at 30 or 37°C before enumeration of CFU on a colony counter (ProtoCOL2; Synbiosis). Triton X-100 treatment did not compromise the ability to form colonies, as tested by plate counting. Adhesion capacities were calculated as (CFU adhered/CFU added) × 100.

Assaying bacterial adhesion to mucin

Microtitre plate wells (Maxisorp Nunc) were coated with mucin (partially purified type III porcine gastric mucin; Sigma Aldrich) as described by Tallon *et al.*[26]. Bacterial suspensions were prepared, as described above, and 1×10^6 CFU were added per well. After 1 h incubation at 37°C, unbound bacteria were removed by washing four times with PBS. Adhered bacteria were detached using 25 μl 0·25 % trypsin/well at 37°C for 10 min with slow shaking, and further suspended in 225 μl PBS to enable plating. Determination of the number of adhered bacteria and calculation of adherence capacity were performed as described above.

Generation of monocyte-derived dendritic cells and immunomodulation

Monocytes were separated from buffy coats obtained from healthy volunteers according to institutional guidelines (Ostfold Hospital Trust, Norway). Peripheral blood mononuclear cells were separated by density gradient centrifugation using Lymphoprep medium (specific gravity 1·077 g/ml; Fresenius Kabi). $CD14^+$ cells were selected using human CD14 MicroBeads (MACS Miltenyi Biotech) and seeded in twenty-four-well plates. $CD14^+$ monocytes were differentiated to immature DC using GM-CSF (50 ng/ml) and IL-4 (25 ng/ml) (both from ImmunoTools). Cytokines were replenished at day 4. The immature DC were treated with UV-inactivated bacteria (60 min UV exposure) for 24 h on day 6. Successful inactivation of bacteria was confirmed by plating the final suspension on agar plates. A cocktail of lipopolysaccharide (100 ng/ml; Sigma-Aldrich), TNF-α (15 ng/ml; ImmunoTools) and PGE_2 (5 μg/ml; Sigma-Aldrich) was used as a positive control for DC maturation[27]. Finally, DC were labelled using phycoerythrin-Cy 5-conjugated mouse anti-human CD83 and Alexa Fluor 700 mouse anti-human CD86 antibodies (MACS Miltenyi Biotech), fluorescence recorded using a MACSQuant flow cytometer and data analysed with MACSQuantify software (Miltenyi Biotech).

Statistical methods

Statistical significance was evaluated using one- or two-way blocked ANOVA models. *P* values were calculated with *t* tests with correction for multiple hypothesis testing or Tukey's test by employing the open source statistical language and environment R (www.r-project.org).

Results

Bacterial cultivability and survival in acidic environments

The ability to multiply in acidic environments (pH 6, 5, 4 and 3) was assayed for different *E. faecalis* strains (Table 1) as an initial screen for acid tolerance. All strains showed a successive decrease in cultivability as the pH was reduced, and none was able to grow at or below pH 4 (data not shown). As this indicates a cut-off value between pH 5 and pH 4, cultivability was next assayed at pH 4·5. Symbioflor-1 was clearly the least acid-tolerant strain when growth at pH 4·5 was compared with growth at neutral pH (Fig. 1(a)). As cell survival after transit through the acidic environment in the stomach is more physiologically relevant than

cultivability in acidic environments, we next assayed cell viabilities following incubation at pH 3 for 30 min. Cell viabilities were markedly reduced for all strains, as indicated by a shift from green (SYTO9) towards red (PI) fluorescence (Fig. 1(b)). Untreated cells were stained in each experiment to confirm intact cell membranes, indicating viable cells (data not shown). Impaired viability was most pronounced for Symbioflor-1, while V583 was least affected. Isolates 62, 158B and LMGT3208 showed intermediate tolerance to the treatments (158B and LMGT3208 not shown). These observations were reinforced by fluorescent microscopy imaging, mainly implemented for visual reference (Fig. 1(c)).

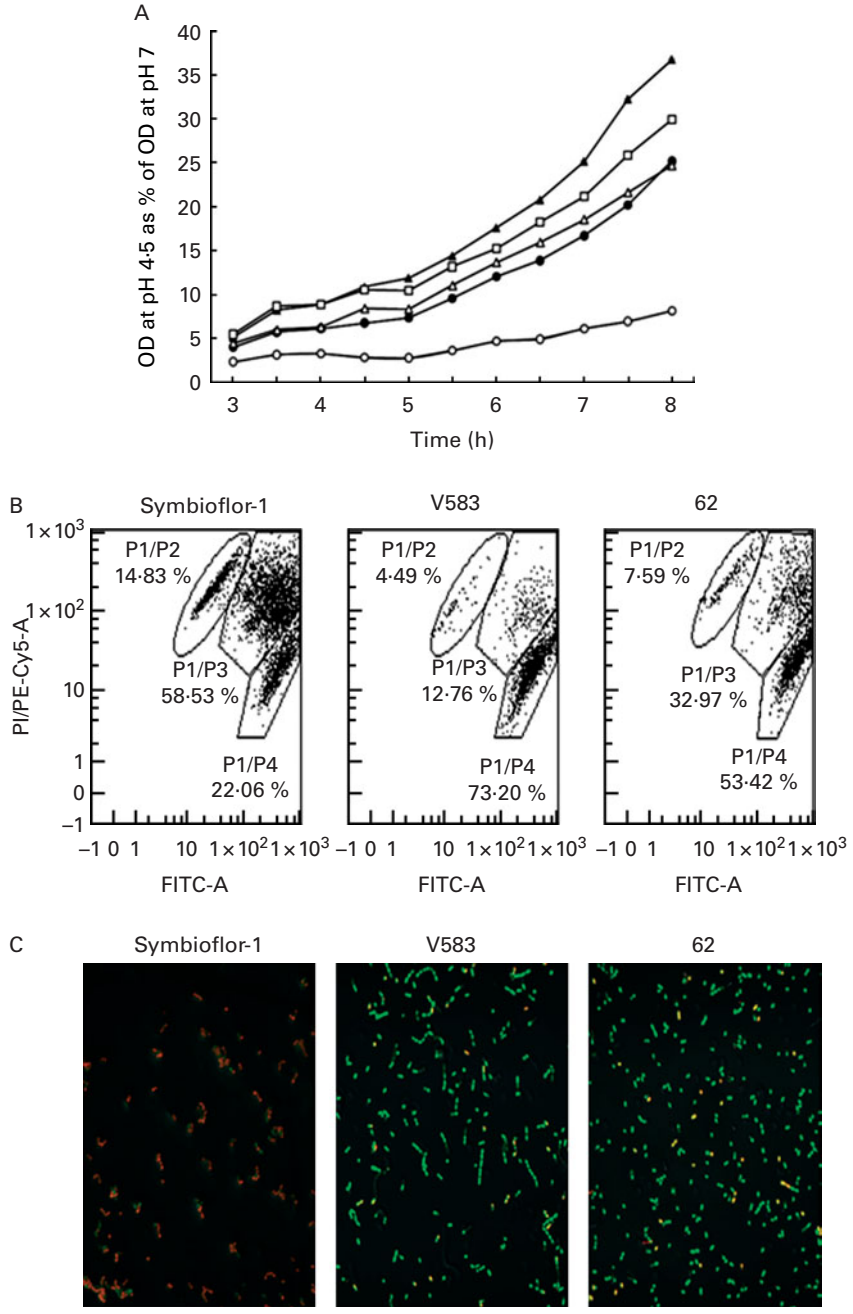

Fig. 1. Acid tolerance of *Enterococcus faecalis* strains. (a) Bacterial growth (optical density (OD) at 600 nm) of Symbioflor-1 (–○–), V583 (–●–), 62 (–Δ–), 158B (–▲–) and LMGT3208 (–□–) in brain–heart infusion (BHI) medium at 37°C with pH 4·5 plotted as percentage of growth at pH 7 against time. Samples were assayed in triplicate and average values plotted after background correction. The data are representative of four different experiments. (b) Flow cytometric analysis of bacterial cultures incubated in BHI medium (pH 3) for 30 min at 37°C subsequently stained with SYTO9 and propidium iodide (PI). A total of 10 000 events were analysed per sample and non-cellular background fluorescence was gated out using the respective side scatter/forward scatter (SSC/FSC) plots (P1). A further gating strategy was used to indicate subpopulations representing dead (P1/P2), sub-lethal (P1/P3) and viable (P1/P4) cells: the percentages of these cells are shown. The dot-plots are representative of at least five experiments. PE, phycoerythrin; FITC-A, fluorescein isothiocyanate-albumin. (c) Fluorescent images were captured of cells treated and stained as described in (b) using a Zeiss LSM 700 confocal microscope equipped with ZEN2010 software.

Bacterial viability after exposure to human gastric and duodenal juices

Bacteria were treated with HGJ (pH 1·5) for 30 min in order to mimic transit through the stomach. This treatment had a detrimental effect on the viability of all strains (Fig. 2(a)). The pH of HGJ was raised to 3·0 (using NaOH) in order to study potential effects of HGJ components other than the extremely low pH. Adjusting the pH to 3·0 enables comparison with the data obtained using media only with pH 3·0 (Fig. 1(b) and (c)), and ensures that digestive enzymes are still active. Viabilities were severely affected after treatment with HGJ (pH 3·0) (Fig. 2(b)), although not to the extent observed after treatment with HGJ with pH 1·5. No strain-specific differences were observed after treatment with either HGJ with pH 1·5 or HGJ with pH 3·0. To mimic transit through the duodenum, bacteria were treated with HDJ for 30 min. In this case, cell viabilities were only affected to a minor extent (Fig. 2(c)). Strain-specific differences were observed, and cells with impaired viability were quantified by gating the relevant subpopulations. Results from three different experiments showed that Symbioflor-1 was the most resistant to HDJ treatment, where only 9·1 % of the cells exhibited impaired viability, while LMGT3208 was the most susceptible strain, with 23·5 % affected.

Adhesion capacity to intestinal epithelial cells and mucin

Adhesion to Caco-2 cells was assayed for all the *E. faecalis* strains (Table 1) and compared with the adhesion of *L. reuteri* DSM 20 016[24] and *L. plantarum* NC8[25], previously reported to bind Caco-2 cells with high and low capacities, respectively[28]. Only 5·1 % of the added Symbioflor-1 cells adhered to IEC, which is significantly less ($P<0·01$) than the adhesion capacities of isolate 62 (14·5 %), 158B (12·3 %) and LMGT3208 (10·6 %) (Fig. 3(a)). The adhesion capacity of Symbioflor-1 was comparable with that of *L. plantarum* NC8 (5·9 %). The same relative species-specific adhesion capacities were found when adhesion to mucin was assessed. Isolate 62, 158B and LMGT3208 bound significantly better to mucin ($P<0·01$) than Symbioflor-1. Intermediate and rather poor adhesion capacities were observed for V583 to Caco-2 cells and mucin, respectively. Cells adhered in general better to Caco-2 cells than to mucin.

Binding competition assay using heparin

A competitive-binding assay was employed to investigate the potential role of glycosaminoglycans as host cell receptors, as previously described for *E. faecalis*[29]. Caco-2 cells were pre-incubated with or without heparin (500 μg/ml)

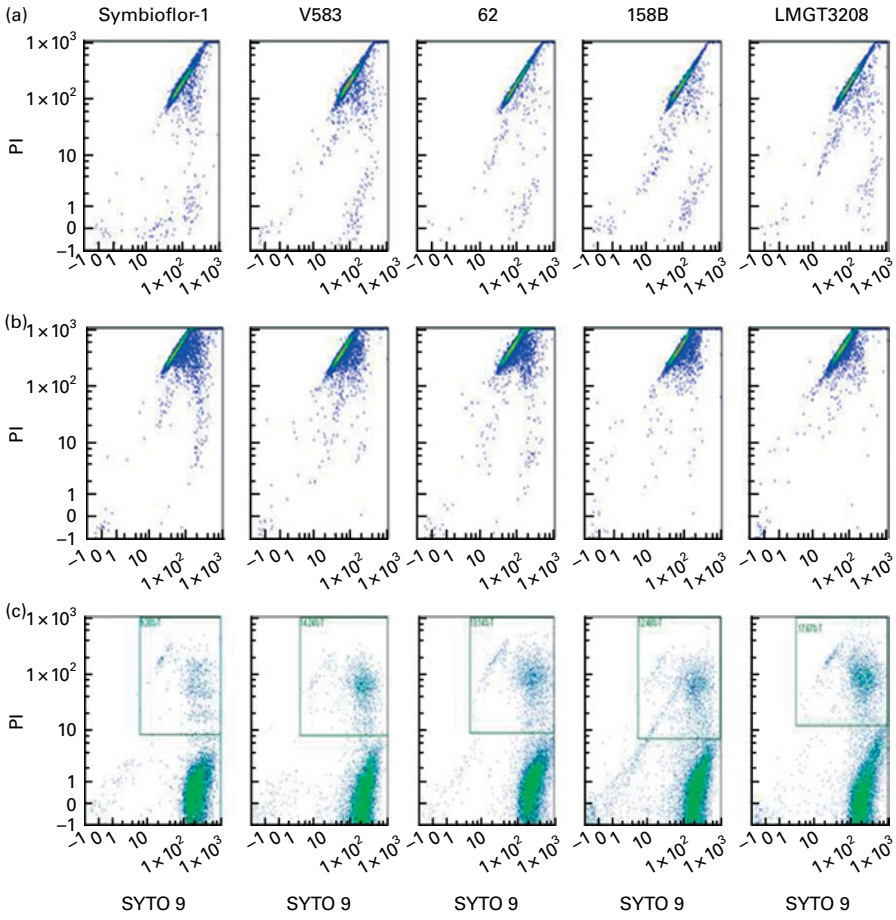

Fig. 2. Tolerance of *Enterococcus faecalis* strains to human gastric juice (HGJ) and duodenal juice (HDJ). Bacterial cultures were treated for 30 min at 37°C with (a) HGJ (pH 1·5), (b) HGJ (pH 3) and (c) HDJ (pH 8). All samples were stained with SYTO9 and propidium iodide (PI) and analysed by flow cytometry. A total of 10 000 events were analysed per sample. Density-plots are representative of three independent experiments.

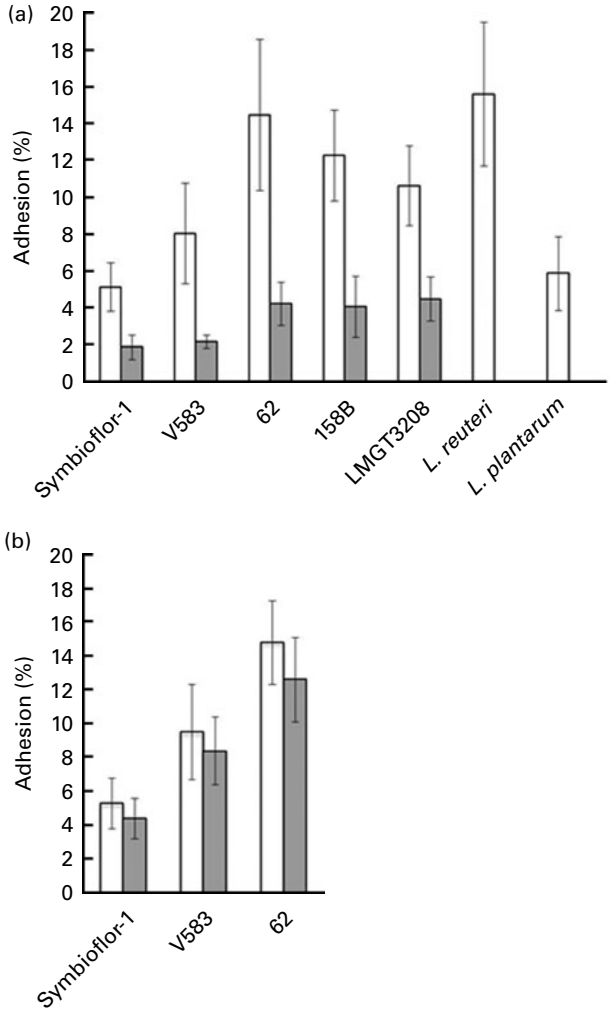

Fig. 3. Adhesion capacities of *Enterococcus faecalis* strains. (a) Wells containing monolayers of Caco-2 cells (□) or mucin (■) were infected with different strains of *E. faecalis*. After 1 h, non-adhered cells were washed off and adhered bacteria solubilised and enumerated by plate counting. *Lactobacillus reuteri* DSM 20 016 and *L. plantarum* NC8 were included as reference strains for high and low binding to Caco-2 cells, respectively. The amount of adhered cells is presented as the percentage of total cells added. All samples were assayed in triplicate and experiments were performed from three to six times. Data are means from three different experiments, with standard deviations represented by vertical bars. (b) Adhesion was assayed with (■) or without (□) heparin (500 μg/ml) as described in (a).

(Sigma-Aldrich) for 30 min before infection and binding to Caco-2 cells was assessed for Symbioflor-1, V583 and 62, as described above. A statistically significant reduction in adhesion ($P<0{\cdot}01$) was found in the presence of heparin (Fig. 3(b)). Heparin appeared to have the same effect for all strains.

Ability of different Enterococcus faecalis *strains to elicit dendritic cell maturation*

The ability of Symbioflor-1, V583 and 62 to mature DC was assayed by monitoring $CD83^+$ and $CD86^+$ cells using flow cytometry. CD83 and CD86 are well-described maturation markers for DC[7,30–32]. An increase in $CD83^+$ and $CD86^+$ cells was observed for all strains as compared with the negative control (Fig. 4). However, no strain-specific differences in the ability to mature DC were detected.

Discussion

The object of the present study was to study several characteristics of Symbioflor-1, commonly assayed when screening bacteria for potential probiotic properties, in comparison with other *E. faecalis* strains (Table 1). Tolerance towards the different environments encountered during GIT transit, adhesion to intestinal cells or mucosa, and the ability to elicit an immune response are likely to be desirable traits[33,34]. *In vitro* methods were employed to address these subjects according to the FAO/WHO recommendations[8].

When ingested, probiotics must overcome environmental barriers in the upper GIT in sufficient numbers and arrive in the intestine in a state able to confer beneficial health effects. The physiological fitness of microbes after simulating exposure to environments in the upper GIT is therefore commonly assayed when screening for probiotic properties[34–38]. The extreme acidity in the stomach (pH 1·5–3·0), in combination with gastric enzymes, is thought to represent a main challenge for survival. Acid tolerance is therefore a highly desirable probiotic trait. It was rather surprising that Symbioflor-1 was most susceptible to acid in comparison with other *E. faecalis* strains, as this microbe has been reported to confer probiotic properties *in vivo*[15,16]. Poor acid tolerance was observed for Symbioflor-1, both when assessing cultivability in acidic environments and viability after simulating transit through the stomach using media with low pH.

Treated cells separated into three different subpopulations when assessed using flow cytometry (Fig. 1(b)). These subpopulations represent dead (high PI/low SYTO9), sub-lethal (high PI/high SYTO9) and live (low PI/high SYTO9) cells, in accordance with previous reports[39,40]. Such distribution patterns contain valuable information about the physiological fitness of bacterial cells, which is an advantage compared with conventional plating[39]. Plating only enables the enumeration of cells able to form a colony, and fails to provide any information about metabolically active cells incapable of cellular division (i.e. forming a colony). Such cells are referred to as 'active but non-culturable' (ANC) cells[41], and are important populations to describe following injury or stress in different aspects of food microbiology.

Human GIT juice is relatively difficult and costly to obtain. As a result, few studies have employed human juices when assessing GIT transit survival[37,38]. Del Piano *et al.*[42] found that several probiotic strains were more sensitive to bovine bile, commonly used in artificial solutions, than to human bile. However, it has also been reported that the sensitivity of probiotics to artificial and human pancreatic juice is very comparable[37]. We exposed the *E. faecalis* strains to HGJ and HDJ in order to study strain-specific tolerance. However, the detrimental effects of HGJ treatments made comparison difficult as all cells were detected in the most compromised subpopulation after treatment (Fig. 2(a)). When using HGJ

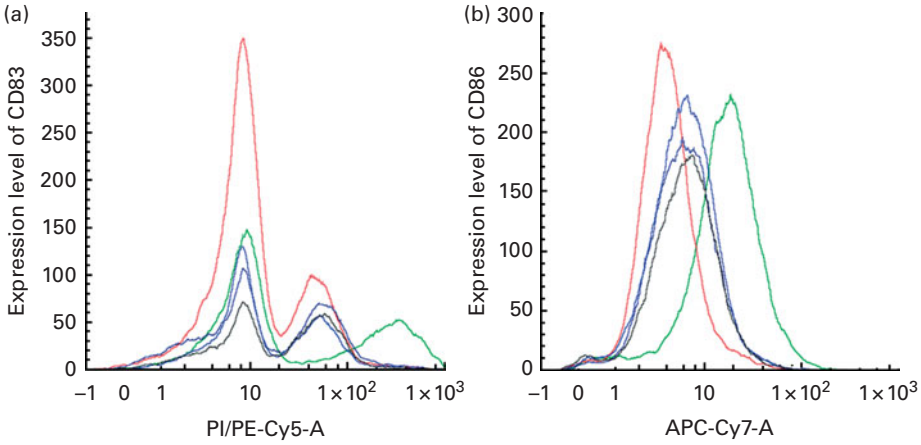

Fig. 4. Ability of different *Enterococcus faecalis* strains to elicit dendritic cell maturation. Immature dendritic cells were treated with UV-inactivated Symbioflor-1 (black), 62 (blue), V583 (light blue), or a cocktail containing lipopolysaccharide–PGE_2–TNF-α (green) and compared with untreated cells (red). Expression levels of CD83 and CD86 were analysed using a phycoerythrin (PE)-Cy5-conjugated anti-CD83 antibody and an Alexa Fluor 700-conjugated anti-CD86 antibody by flow cytometry. These results are representative of three different experiments with parallel samples. PI, propidium iodide; APC, allophycocyanin.

where the pH had been raised to 3, minor improvements of physiological fitness were observed (Fig. 2(b)). Nevertheless, the cell viabilities were still affected to such an extent that strain-specific differences could not be detected using gating strategies on the dot-plots. These results show that both the extremely low pH, but also other components of the HGJ, such as digestive enzymes, are efficient in killing the *E. faecalis* strains tested in the present study if orally ingested.

Using neat HGJ reflects the gastric environment of a fasting individual more so than in a normal *in vivo* situation where the gastric ventricle also contains a food matrix. Food and especially milk-based products are known to have a buffering capacity that consequently will protect ingested microbes from the lethal effect of HGJ[34,39]. The loss of viability reported in the present study is therefore expected to be higher than in a normal *in vivo* situation. Nevertheless, in view of the observed susceptibility to acidic environments, Symbioflor-1 might have a better chance of surviving transit through the stomach if implemented in a milk-based product, encapsulated or somehow protected against this harsh environment[39,43]. Currently Symbioflor-1 is administrated as drops.

Some model systems simulating GIT transit involve the sequential immersion of microbes in gastric and duodenal solutions[34–36,38,44]. As the effect of HGJ treatment on the *E. faecalis* strains in our experiments was so pronounced, no strain-specific differences would have been observed with such a sequential model. We therefore assessed the effects of HGJ and HDJ treatments separately. In contrast to HGJ treatment, all strains survived exposure to HDJ relatively well (Fig. 2(c)). This suggests that transit through the gastric ventricle is the main determining factor for survival in the upper GIT for the strains studied. Strain-specific differences were observed after HDJ treatments, and Symbioflor-1 was identified as the most resistant strain. Bile acids are known as a major stress factor in the duodenal area; however, protecting mechanisms such as the synthesis of stress proteins and swift adaptive abilities have been reported for *E. faecalis* and might explain the high survival rate following HDJ treatment[45–47]. However, the *in vivo* significance of this observation must be questioned, as the viability of Symbioflor-1 would already have been severely compromised in the preceding transit through the stomach.

Nueno-Palop & Narbad[35] included ten *E. faecalis* strains in a study assessing probiotic properties of bacteria isolated from human faeces. Only one of these *E. faecalis* strains, CP58, was able to survive a simulated *in vitro* digestion assay in adequate amounts. This highlights the existence of strain-specific variation in regards to GIT transit tolerance. More than 40 % of *E. faecalis* CP58 survived the digestion assay, which is considerably higher than the survival of Symbioflor-1 reported in the present paper. However, as milk was included in the media when assessing CP58, the relatively higher survival rate might be attributed to the well-described buffering effect of milk[34,39].

One may speculate whether the differences in test performance between Symbioflor-1 and the other *E. faecalis* strains are due to the reported absence of large genomic regions from the chromosome of Symbioflor-1, indicating gene loss[20]. Perhaps important determinants of acid tolerance or adhesion, such as proton pumps or surface proteins, have been lost from the genome of Symbioflor-1. However, genes encoding determinants such as aggregation substance, collagen adhesion protein, and the ability to resist oxidative stress were detected[20], implying that Symbioflor-1 is somewhat equipped for exhibiting probiotic traits.

Whether intestinal adhesion is a prerequisite for probiotic cells is debatable. Nevertheless, adhesion will reduce the limiting effects of peristalsis on intestinal residence time, and may block pathogen attachment sites. Adhesion is therefore commonly addressed when screening for probiotic strains *in vitro*[35,48,49]. We investigated the adhesion capacities of the *E. faecalis* strains to IEC using the well-established Caco-2 cell line[50–52]. Culture-related conditions may lead to variations between cell cultures maintained in different laboratories, which makes it difficult to compare results in the literature[50,53]. To ensure that the adhesion data were not biased due to a differentiated cell line, we performed experiments using cells maintained in two different laboratories.

The resulting adhesion data correlated well, and were comparable with previous reports[28,54,55]. We found that Symbioflor-1 bound no better to IEC than the reference strain included for its low binding capacity (Fig. 3(a)).

The use of several different methods when studying bacterial adhesion to the intestine *in vitro* has been recommended in order to corroborate observations and perhaps elucidate the type of interactions and molecules that mediate the bacteria–host interaction[33,55]. Furthermore, the model using Caco-2 cells has clear limitations, such as the lack of mucus secretion. We therefore expanded the adhesion studies by assessing binding capacities to mucin. Mucins are polymeric glycoproteins found in mucus and have been widely used for assaying intestinal adhesion of bacteria[26,55–59]. The strain-specific adhesion capacities to mucin correlated well with our Caco-2 data and therefore support the observation that Symbioflor-1 has a rather poor adhesion capacity *in vitro*.

As mentioned above, variations in experimental protocols and in the maintenance of cell lines may cause deviations in adherence capacities for a given strain. Comparisons with other studies should therefore be done with great care. However, the percentage of added bacteria that adhered to the substratum in the present study correlates well with other reports.

In the present study the adhesion to mucin was generally lower than the adhesion to Caco-2 cells (Fig. 3(a)). Reported bacterial adhesion capacities to Caco-2 cells in comparison with mucin have been variable[33,55]. Tuomola *et al.*[33] assayed the adhesion of six different commercial probiotic strains to Caco-2 cells and mucin and found great variability in whether the strains adhered best to cells or mucin. Laparra & Sanz[55] observed better adhesion of probiotics to mucin than to Caco-2 cells. Also, different variants of mucin are employed in these studies, complicating the comparison of results.

A higher affinity for Caco-2 cells, as compared with mucin, made it tempting to speculate that the *E. faecalis* strains adhere to one or several specific components on the intestinal cell surface. Glycosaminoglycans, ubiquitously expressed on the surface of mammalian cells, represent attachment sites for bacteria, including strains of *E. faecalis*[60–62]. Sava *et al.*[29] provided evidence for the involvement of heparin in adhesion to Caco-2 cells for some, but not all, of the *E. faecalis* strains tested. Heparin has also been shown to block the binding of *Escherichia coli* O157:H7 to colonic epithelial cells[63]. We speculated whether the poor adhesion capacities of Symbioflor-1 was a result of this strain being deficient in a putative surface molecule which normally confers binding to host receptors, such as glycosaminoglycans. The fact that an absence of large genomic regions has been reported for Symbioflor-1 in comparison with other *E. faecalis* strains[20] strengthens this hypothesis. We therefore performed competitive binding assays using heparin in order to investigate such potential molecular mechanisms which might be responsible for the differences in Caco-2 adhesion observed for the different *E. faecalis* strains studied in the present experiment. Heparin was found to significantly reduce the binding of the strains tested ($P<0{\cdot}01$), which indicates that that glycosaminoglycans might be involved in the bacteria–host interactions. However, no significant differences in the effect of heparin on the strains with (Symbioflor-1) and without (V583 and 62) gene loss were observed (Fig. 3(b)).

DC are antigen-presenting cells with a pivotal role in regulating the immune system at mucosal surfaces[64]. Immature DC reside in peripheral tissues, such as the gut mucosa, and continuously sample the microenvironment via pattern recognition receptors (PRR), such as the Toll-like receptors[65]. Specific interactions between bacteria and PRR trigger DC maturation, and, importantly, modulate DC differentiation in such a way that both tolerance towards commensals and generation of protective immune responses against pathogens are enabled[64]. Bacteria, including probiotics, differ in their immunomodulatory activity towards DC maturation and may therefore influence the polarisation of immune responses[66–68]. We exposed immature DC to different *E. faecalis* strains to (i) establish whether these bacteria were able to induce DC maturation and (ii) to identify potential strain-specific differences. All strains tested increased the number of $CD83^+$ and $CD86^+$ cells as compared with untreated cells (Fig. 4). No strain-specific differences were observed, which is somewhat surprising considering the highly diverse *in vivo* effects of the probiotic, pathogenic and commensal strains. Inactivated bacteria are commonly used in similar experiments[7,66,69], which is probably due to the presumption that bacterial cell-surface molecules are considered the key factors in bacteria–DC communication via the PRR[5]. However, one must not rule out other paths of communication between bacteria and the immune system such as via IEC. Substantial evidence has been provided for differential cross-talk between IEC, DC and bacteria involved in the regulation of intestinal homeostasis[67,70–72]. The possibility of a greater maturation potential by metabolically active bacteria *in vivo* can therefore not be ruled out.

According to the results presented in the present paper, acid tolerance and/or intestinal adhesion do not appear to be crucial factors involved in the beneficial effects of Symbioflor-1 on host health. This implies that *in vitro* observations may not always reflect the *in vivo* situation. In support of this, clinical studies have documented beneficial health effects of the well-studied probiotic strains *L. rhamnosus* GG and *L. plantarum* 299v[73,74]. Nevertheless, these strains did not perform well in a recent study using *in vitro* methods[28]. Vice versa, *in vitro* studies of *L. plantarum* MF1298 showed excellent *in vitro* probiotic properties[75,76], but this bacterium was later reported to exhibit unfavourable effects on symptoms in subjects with irritable bowel syndrome[77]. In conclusion, *in vitro* testing is a rational starting point for selecting microbes with a probiotic potential or to establish possible mechanisms of action, but not adequate to predict the functionality of probiotic micro-organisms in the human body. In line with the recently expressed opinions of Rijkers *et al.*[78], it must be emphasised that human clinical trials are the definitive tool to establish probiotic status.

Acknowledgements

The present study was supported by The Norwegian University of Life Sciences, Norway and Østfold University College,

Norway. T. E. C. was involved in experimental procedures and data analysis of all experiments, and wrote the manuscript. H. J. performed the adhesion experiments to mucin, some statistical analysis and contributed to discussions. C. R. K. contributed to experimental designs of flow cytometric analysis and discussions. G. D. performed statistical analysis. M. J. ensured access to human gastrointestinal juices and contributed to the experimental design of related experiments. T. L. supervised the project. All authors contributed to the critical revision of the manuscript. There are no conflicts of interest to declare.

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