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Impact of extensively hydrolyzed infant formula on the probiotic and postbiotic properties of *Lactobacillus fermentum* in an *in vitro* co-culture model†

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Early gut homeostasis is a balance between dietary antigen exposure, gut barrier function, microbiome establishment and orchestration of innate and adaptive immune responses. Imbalances during this early time of development can lead to increased susceptibility to immune reactions like allergy. Especially for infants with a predisposition to allergies and who cannot be exclusively breastfed, there are different human milk substitutes, including hydrolyzed infant formula, which are supposed to prevent allergy-associated mechanisms. The physiologic mechanism beyond the destruction of cow's milk allergenic structures in those formulas are currently not fully understood. Therefore, our aim was to elucidate the impact of hydrolyzed infant formula on intestinal homeostasis and presumed mechanisms behind the beneficial effects. In this study, we used a triple co-culture *in vitro* model of gut inflammation and homeostasis, including enterocyte-, goblet- and macrophage-like cells in a transwell setup, to assess the effect of extensively hydrolyzed (eHF) infant formula compared to standard cow's milk-based infant formula with intact protein (iPF). These formulas were combined or not with heat-inactivated *Limosilactobacillus fermentum* CECT 5716 (*Lf*) to test the effect of probiotic compounds in combination with different types of infant formula (*i.e.* eHF and iPF) on the intestinal barrier and cytokine production. Under LPS-inflammatory trigger, eHF and eHF + *Lf* increased mucus production and MUC2 mRNA expression, restored epithelial barrier integrity and increased secretion of regulatory TGFβ, compared to respective controls. These results suggest a beneficial role for eHF, and especially eHF + *Lf*, in restoring intestinal homeostasis and attenuating pro-inflammatory responses.

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Introduction

The intestinal epithelium is essential for regulation of nutrient absorption and maintaining intestinal homeostasis. It facilitates a coordinated interaction among intestinal epithelial cells, microorganisms, and the immune cells beneath, thereby modulating innate immune functions and responses to antigens. Infancy represents a crucial period for immune programming and the induction of oral tolerance. During this critical phase, the initial exposure to environmental triggers, along with age-specific mechanisms governing dietary antigen exposure and the translocation of macromolecules across the maturing intestinal barrier, contributes to the establishment of oral tolerance to food antigens.[1]

Exclusive breastfeeding is the preferred and recommended method of infant feeding for the first six months of life.[2–4] Human milk (HM) provides the best nutrition for infants, delivering essential nutrients and promoting the development of a healthy gut microbiota through its probiotic and prebiotic components.[2,5] As a result, breastfed infants typically have gut bacteria profiles with a greater presence of the *Bifidobacterium* spp. and lower overall microbial diversity.[6,7]

Adequate infant gut development is characterized by a homeostasis between dietary antigen exposure, gut barrier function, gut microbiota maturation and orchestration of

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innate and adaptive immune responses.[8] Disturbances in this process can lead to increased susceptibility to allergy and inflammation processes, which is seen by the fact that breastfed infants have lower risk for *e.g.* infections.[9]

When exclusive breastfeeding is not possible, and the infant is at higher risk for allergy development (*e.g.* by genetic predisposition and environmental factors) several guidelines recommend to avoid feeding an infant formula with intact cow's milk protein and mention formula with extensively hydrolyzed formula (eHF) as one possible substitute.[10] Following HM composition, specific -biotic compounds (pre-, pro- and postbiotics) can be added to infant formula for their assumed health promoting effect.[11] In this study, we focused on the probiotic strain *Limosilactobacillus* (*L.*) *fermentum* CECT 5716 (*Lf*), originally isolated from HM, with putative health promoting effects that qualify the strain as probiotic.[12–15] *Lf* in combination with prebiotic galactooligosaccharides (GOS) increased the abundance of bifidobacteria and improved markers for stool softness.[16,17] In a recent study, protein modification in infant milk formula has been associated with changes in probiotic metabolic activity and bacterial composition.[18] In infant formula, *Lf* was shown to reduce the incidence of diarrhoea, and a recent study by Piloquet *et al.* could show the effectiveness in reducing the incidence of lower respiratory tract infections.[19,20]

The effect of *Lf* in different infant formula matrices on the respective formulas' allergenic activity was tested in an *in vitro* basophil activation experiment. This experiment revealed that *Lf* in an extensively hydrolyzed protein infant formula matrix is capable to reduce basophilic degranulation compared to the same formula without *Lf*. This allergenic-activity reducing effect of *Lf* was not observed in an intact protein based infant formula matrix. Hereby it can be seen that *e.g.* the protein matrix can have a great effect on the probiotic properties.[21]

In the current study, our main aim was to investigate the effect of extensively hydrolyzed infant formula (eHF) alone or combined with heat-inactivated *L. fermentum* CECT 5716 (*Lf*) on the intestinal barrier integrity and cytokine production. Furthermore, matrix specific effects should be assessed by comparing with infant formula based on intact cow's milk protein (iPF), both containing GOS. To mimic the infant intestine, we used a triple co-culture *in vitro* model of gut inflammation and homeostasis, including enterocyte-, goblet- and macrophage-like cells in a transwell setup. In addition, we presented the effect of simulated gastrointestinal digestion on a number of assays as a complement to the *in vitro* approach.

Materials and methods

Bacterial strain and formula matrices

The probiotic strain *L. fermentum* CECT 5716 (*Lf*) was grown in de Man, Rogosa and Sharpe (MRS) agar (Oxoid, Spain) and incubated for 18 hours at 37 °C under anaerobic conditions.

Heat-inactivated *Lf* (65 °C for 15 min) was prepared from 10 ml suspension of *Lf* (10^8 CFU per mL) in MRS centrifuged at 7000 *g* for 10 min, and suspended in 10 ml of phosphate buffered saline (PBS). Eight serial dilutions of the suspension were plated in triplicate in MRS agar plates and incubated 48 hours in anaerobic conditions at 37 °C to ensure inactivation effectiveness. No growth was observed in any of the dilutions. For further assays, heat-inactivated *Lf* was diluted in the corresponding media to obtain a final concentration of 10^6 cells per mL, estimated from optical density and plate counting of the original *Lf* suspension.

Infant formula matrix setups included intact protein formula (iPF; HiPP Pre Bio Combiotik®, liquid) and extensively hydrolyzed formula (eHF; HiPP Pre HA Combiotik®, liquid), both supplemented with prebiotics (Galactooligosaccarides GOS, 0.3 g per 100 mL) (ESI Table S1†). HiPP HA infant formula was manufactured from Peptigen® IF-3080 (supplied by Arla Foods Ingredients, Videbæk, Denmark) which is suitable as sole protein source in infant formulas.[22,23] Infant formula samples and *Lf* were provided by HiPP GmbH & Co. Vertrieb KG (Germany).

Viable *Lf* was only used for adhesion experiments, whereas inactivated *Lf* (post-biotic) was applied to evaluate the effect on mucus production, epithelial barrier integrity and soluble factors quantification.

Gastrointestinal digestion of infant formula matrix

The infant formula iPF and eHF, supplemented or not with active and heat-inactivated postbiotic. *Lf* at 10^6 cells per mL was digested following the protocol described in Calatayud *et al.*[24] with the adaption towards infant conditions as follows in infants *i.e.*: (i) shortening of the gastric phase to 60 min and final pH of 4.0; (ii) shortening intestinal phase to 60 min and final pH of 6.5; (iii) reducing pepsin levels from 2000 U ml^{-1} to 300 U ml^{-1}; (iv) reducing bile salts to 3 mmol L^{-1}.

In vitro digested samples were diluted 1/10 in supplemented Dulbecco's Modified Eagle's medium, high glucose (DMEM, Gibco, cat. no. 11965092), before use in cell culture assays (passage 3–5). Supplemented DMEM contained 10% Inactivated fetal bovine serum (iFBS); Greiner Bio One, cat no. 758093, 1X antibiotic-antimycotic solution, Gibco, cat. no. 15240062 and 1X GlutaMAX™, Gibco, cat. no. 35050061. ESI Tables S2–S4† provide a schematic representation of assays and conditions applied in this research.

Adhesion of *L. fermentum* CECT 5716 to the triple co-culture *in vitro* gut model

Adhesion assays were performed in 96-well plates containing Caco-2/LS174T cells (50/50). Caco-2 cells (ECACC 86010202) and LS174T cells (ECACC 87060401) were maintained as previously described.[25,26]

Caco-2/LS174T cells were seeded at 1.5×10^5 cells per cm^2 in supplemented cell culture media without antibiotics and maintained 5–6 days post-confluence. Then, fresh *Lf* grown in MRS was normalized to 10^7 bacterial cells per mL and stained with Acridine Orange (AO, Sigma) following the procedure

described in.[27] A final concentration of 10^6 AO-stained bacterial cells was suspended in 1 mL of supplemented DMEM without antibiotics. Then, 100 μL of AO-stained bacterial suspension were added to the Caco-2/LS174T cells and incubated for 2 hours at 37 °C, 95% relative humidity and a 5% CO_2. Then, fluorescence from each well was quantified using a CLARIOstar plate reader [ex/em: 500/526 nm (DNA) and 460/650 nm (RNA)] with an area screening mode covering all the surface of the wells. Subsequently, supernatants containing AO-stained bacteria were transferred to black well plates for fluorescence quantification using the same parameters described above as a quality control. Caco-2/LS174T cells were washed three times with supplemented DMEM without antibiotics. Finally, Caco-2/LS174T monolayers and supernatants were measured as previously described.

Assessment of the effect of infant formula matrix and *L. fermentum* CECT 5716 on mucus production

Caco-2/LS174T cells were grown in a 96-well plate as described in previous sections. Cells were exposed to different infant formula matrices in presence or absence of heat-inactivated *Lf* for 24 hours, from native and digested samples. Mucus quantification was conducted by chemical removal of the cell-associated mucus layer and total protein quantification as previously described.[28] Briefly, supernatants were removed and cells were washed once with PBS (200 μL per well) and subsequently, 100 μL *N*-acetyl-L-cysteine (NAC) in Hanks Balanced Salt Solution (HBSS) (10 mM) was added to the wells and incubated for 1 hour at 37 °C, 10% CO_2 and shaking (60–80 rpm). After incubation time, the NAC supernatant was transferred to a new 96-well plate and cells were washed with 100 μL of HBSS, which was also recovered and transferred to the 96-well plate, pooling the NAC supernatant and the HBSS wash (final volume ≈ 200 μl). Samples were condensed using a SpeedVac, suspended in 50 μL PBS overnight at 4 °C and total protein content was quantified using BioRad Bradford protein assay following the manufacturer instructions. The absorbance was then measured at 595 nm using a CLARIOstar plate reader and mg mL^{-1} of protein were obtained from the blank-corrected absorbance of the samples plotted to the blank-corrected standard curve with bovine serum albumin (0–0.5 mg mL^{-1}).

Triple co-culture *in vitro* gut model

An *in vitro* model of the human gut mucosa was assembled using a 6 well transwell system (pore size 0.45 μm, PET) (corning), growing on a semipermeable membrane a co-culture composed by 90% Caco-2 cells and 10% LS174T cells, seeded at a density of 6.4×10^4 cells per cm^2 in supplemented DMEM. Cells were maintained for 7 days with medium refreshment every 2–3 days. After 7 days, THP-1 cells were seeded separately in 6-well-plates at a density of 1×10^5 cells per cm^2 in RPMI 1640 medium without iFBS, with antibiotics, containing 200 ng mL^{-1} of 12-*O*-tetradecanoylphorbol-13-acetate (PMA, Sigma), to induce macrophage-like differentiation. Plates were incubated for 24 hours at 37 °C, 5% CO_2–95% air in a humidified incubator. After incubation, PMA containing medium was removed and cells were washed twice with RPMI 1640 medium, maintained 5 days with supplemented RPMI 1640 medium with refreshments of medium every 2 days. Morphology was assessed by optical microscopy (Olympus CKX41, Olympus Life Science, Spain) at every refreshment. Transwell inserts containing Caco-2/LS174T co-cultures were then assembled on top of differentiated THP-1 cells and the triple co-culture was maintained for 5–7 days before the assay. In total, co-culture of Caco-2/LS174T cells was maintained for 19 days.

Then, native or digested infant formula matrices diluted 1 : 5 v/v in supplemented DMEM were added to the apical compartment of the transwell inserts containing Caco-2/LS174T co-cultures, simultaneously to sodium dodecyl sulfate (SDS; 20 mM; Sigma) to disrupt the simulated gut epithelial barrier as previously described.[29] SDS was applied to all apical compartments, including control (−) condition. Infant formulas were administered with or without the addition of a proinflammatory stimulus, consisting of 10 ng mL^{-1} of LPS in the basolateral compartment to create a proinflammatory environment (control (+)),[30] and with or without the addition of *Lf* (ESI Tables S2 and S3†). Controls only containing cell culture media without any treatment (blank) were included in different batches. After incubation for 24 hours at 37 °C, 5% CO_2–95% air in a humidified incubator, epithelial barrier integrity was assessed and apical and basal media were recovered and immediately stored at −80 °C.

In each assay, a quality control of cell viability was performed using resazurin reduction method as previously described[31] (see also ESI†). None of the treatments induced cytotoxicity and only results with cell viability above 80% were detected.

Epithelial barrier integrity assessment using Lucifer Yellow and TEER measurement

To assess epithelial barrier integrity after cell exposure to native or digested infant formula matrices, Lucifer Yellow (LY, 100 μM, Sigma) transport was assessed as previously described.[28] LY was added in apical compartments of the transwell system. Right after addition, a 100 μL aliquot was transferred to a black 96 well plate from both apical and basolateral compartments, to obtain a baseline value. After one hour of incubation, another aliquot of 100 μL was taken from each well, both from apical and basolateral compartments, and transferred to a black 96 well plate. Fluorescence was then measured at 536–20 nm excitation (428–15 nm) using a CLARIOstar plate reader. To estimate epithelial barrier integrity, percentage of transport was calculated as LY % transport = 100 × (AFU basolateral time X/AFU apical time 0), where AFU are the arbitrary fluorescent units recorded by the plate reader.

Transepithelial electrical resistance (TEER) was measured using a Millicell® ERS-2 Voltohmmeter (Merck). Values of TEER from empty-cells inserts with cell culture media in the apical and basolateral compartment were subtracted from the different measures, and values obtained in ohms (Ω) were cor-

rected by the surface of the transwell (4.67 cm^2) and expressed as $\Omega\ cm^2$.

Measurement of soluble factors: cytokines and intestinal alkaline phosphatase activity

Cytokine production was evaluated using commercial ELISA kits (IL-8 and IL-10 Human Uncoated ELISA Kit, human TGF-β 1 ELISA Kit, and human MIP-3 alpha/CCL20 ELISA Kit Invitrogen Thermo Fisher Scientific) following manufacturer's instructions. Baseline cytokine levels present in the supplemented cell culture media containing FBS were quantified in each experimental batch and subtracted from the cytokine concentrations measured in our experimental conditions. If required, sample dilution was performed with sample dilution buffer to obtain absorbance values within the linear range of the standard curve. Assay sensitivity and assay range for each ELISA kit are reported in ESI Table S5.†

Intestinal alkaline phosphatase (IAP) activity was assessed in the apical and basolateral compartment of the transwell system at the end of the assay.[32] Supernatants (5 μL) were collected and enzymatic activity was quantified using the Alkaline Phosphatase Diethanolamine Activity Kit (Sigma). Colorimetric reaction was quantified in a SpectroStar Nano (BMG Labtech, Ortenberg, Germany) at 405 nm for 15 min, with reads every 2 min. In each run, blank samples containing treatments without cell exposure (*e.g.*, cell culture media, addition of SDS or infant formula matrices), were run to obtain baseline values of IAP activity. IAP activity in units per mL was calculated as follows: IAP = (ΔA_{405} nm per min Test − ΔA_{405} nm per min Blank) (df) (VF)/(18.5) (VE), with df = dilution factor; VF = volume (in mL) of assay; 18.5 = millimolar extinction coefficient of *p*-Nitrophenyl Phosphate (PNPP) at 405 nm; VE = volume (in mL) of sample solution used; blank = each sample was subtracted with the corresponding blank (cell culture media or infant formula matrix and combinations with *Lf*, LPS and SDS).

Gene expression by Real-Time-PCR

RNA was extracted from lysed cells using a commercially available kit for RNA extraction (NucleoSpin® RNA Plus, Macherey-Nagel), following manufacturer instructions. RNA quality was assessed using Nanodrop ND-1000 (Thermo Fisher Scientific), to check samples were between 1.8–2.2 260/230 nm ratios. RNA samples were then reverse-transcribed using a commercial kit (High-Capacity cDNA Reverse Transcription Kit, Thermo Fisher Scientific). Briefly, for each sample 2 μL of 10X RT Buffer, 2 μL of 10X RT OligoDT, 0.8 μL of 25X dNTPs Mix (100 mM), 1 μL of Multiscribe™ Reverse transcriptase, and 4.2 μL of nuclease-free water were mixed to prepare a 2× master mix. Then, 10 μL of each sample were mixed with the same amount of 2× master mix in 0.2 mL PCR tubes. The reaction was performed in a T100™ thermal cycler (Bio-Rad) in three steps: 10 min at 25 °C followed by 120 min at 37 °C and a final step of 5 min at 85 °C. The obtained cDNA was used for RT-PCR analysis in a LightCycler® 480 real-time PCR system (Roche) with a final volume reaction of 10 μL, containing 5 μL of SybrGreen (Roche) reaction buffer, 0.6 μL of reverse and forward primers mix, 150 μM each (Metabion), 3.4 μL of RNase-free water, and 1 μL of sample cDNA. Primers used in this research are described in ESI Table S6.† Data analysis was performed using the Relative Expression Software Tool – Multiple Condition Solver, version 2 (REST-MCS ©)[33,34] actin B was used as a reference gene. Data are reported as log 2 fold-change ± standard error. Otherwise stated, mRNA expression levels were tested *versus* control (−) condition. When different formula matrices were compared, iPF or combinations thereof with *Lf* and/or LPS (*i.e.*, iPF *vs.* eHF; iPF + *Lf vs.* eHF + *Lf*; iPF + *Lf* + LPS *vs.* eHF + *Lf* + LPS), were used as control.

Evaluation of modulatory effects of infant formula matrices and *L. fermentum* CECT 5716 on TLR4-NFKβ activation

HEK-TLR4 reporter cells (purchased from InvivoGen Europe; Tolouse, France) were seeded in 96 well plates at a density of 1.5×10^5 cells per cm^2 in supplemented DMEM High Glucose. Cells were maintained for 24 hours and then the plates were washed twice with supplemented DMEM without antibiotics. Infant formula matrices were diluted 1 : 5 with DMEM (v/v) and the dilutions were added to HEK-TLR4 cells, which were incubated at 37 °C in 5% CO_2–95% air atmosphere in a cell incubator for 16–18 hours.

For the treatments involving infant formula matrices, all treatments including controls were performed in two conditions: presence or absence of lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (Sigma) at 10 ng mL^{-1} and presence or absence of heat-inactivated *Lf*. To preserve the receptors (TLRs) on the cell membranes, no SDS was used in this setup.

After incubation, 5–10 μL of supernatants were transferred from each well to a clear 96 well plate and tested for SEAP (secreted embryonic alkaline phosphatase) activity. After 15 min of incubation at 65 °C to inactivate endogenously produced alkaline phosphatases, 95 μL of PNPP phosphatase substrate (Thermo Fisher Scientific) were added in each well and absorbance was measured at 405 nm every minute for 30 min using a CLARIOstar plate reader (BMG LABTECH GmbH). Data were normalized using protein quantification and expressed as arbitrary fluorescence units (AFU).

Statistical analysis

All statistical analyses and graphs were performed using GraphPad Prism 8.0.2. (GraphPad Software, Boston, MA, USA). One-way or two-way ANOVA were performed followed by Tukey's HSD or Dunnet's *post hoc* test. Significance was considered at the 5% level ($\alpha = 0.05$).

Results

Adhesion of viable *L. fermentum* CECT 5716 to the intestinal epithelium is influenced by infant formula matrix

The infant formula matrices, *i.e.* the form of protein (intact or hydrolyzed), influenced the adhesion of *Lf* to the simulated

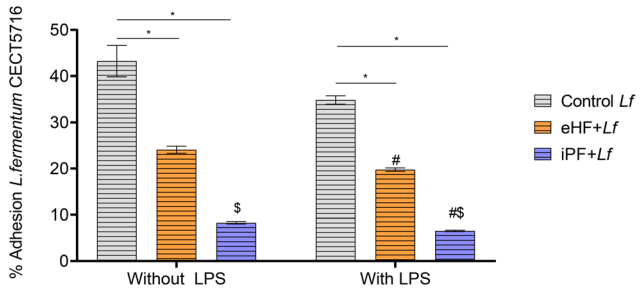

Fig. 1 Effect of infant formula matrices on the adhesion of viable probiotic *L. fermentum* CECT 5716 to the intestinal epithelium. Bars represent the percentage (mean ± standard deviation, n = 6) of *L. fermentum* CECT 5716 adhesion in presence of different infant formula matrices without or with LPS (10 ng mL^{-1}). Significant differences between Control + *Lf* and different matrices are marked with an asterisk (* $p < 0.05$). Significant differences between LPS or non-LPS treatments within the same infant formula matrix are marked with hash symbol (# $p < 0.05$). Significant differences between different matrices (*i.e.*, iPF *vs.* eHF; iPF + *Lf vs.* eHF + *Lf*; iPF + *Lf* + LPS *vs.* eHF + *Lf* + LPS) are marked with a dollar symbol ($ $p < 0.05$). LPS = lipopolysaccharide; eHF = extensively hydrolyzed formula; iPF = intact protein formula.

epithelium (Fig. 1). Treatment with LPS significantly reduced *Lf* adhesion in all formula-treated conditions (# $p < 0.05$), compared to non-inflamed epithelium. Significantly higher adhesion levels ($ $p < 0.05$) were shown for eHF (24 ± 1.9%), compared to iPF (8.3 ± 0.6%), irrespective of inflammatory conditions.

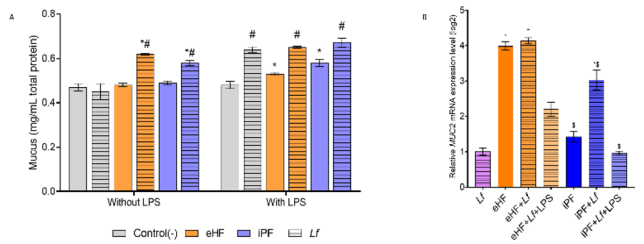

Fig. 2 Effect of infant formula matrices and postbiotic *L. fermentum* CECT 5716 on mucus production and MUC2 mRNA expression. (A) Bars represent the percentage (mean ± standard deviation; n = 6) of total protein content of recovered mucus from co-cultures of Caco2/LS174T cells in presence of different infant formula matrices without (full colour) or with (lined bars) *L. fermentum* CECT 5716 (*Lf*). Significant differences between respective control (–) (with or without *Lf*) within each LPS-condition (without or with LPS) are marked with an asterisk (* $p < 0.05$). Significant differences within the same matrix with *vs.* without *Lf* (*i.e.* control (–) *vs.* control (–) + *Lf*; eHF *vs.* eHF + *Lf*; iPF *vs.* iPF + *Lf*) within the same condition (with or without LPS) are marked with a hash symbol (# $p < 0.05$). Statistical analysis associated to this graph is presented in ESI Table S9.† (B) Bars represent expression ratio (2 log units) (mean ± standard deviation; n = 6) of MUC2 mRNA expression in the simulated intestinal epithelium (Caco2/LS174T). Relative expression was calculated against control (–) condition and significant differences are marked with an asterisk (* $p < 0.05$). Significant differences between different matrices (*i.e.*, iPF *vs.* eHF; iPF + *Lf vs.* eHF + *Lf*; iPF + *Lf* + LPS *vs.* eHF + *Lf* + LPS) are marked with a dollar symbol ($ $p < 0.05$).

L. fermentum CECT 5716 induces mucus production *in vitro*, independent of protein matrix

In absence of LPS (*i.e.*, non-inflamed simulated epithelium), infant formula matrices had no effect on mucus production, compared to cell culture medium (Fig. 2A, full coloured bars). *Lf* alone increased mucus production in presence of LPS, while had no effect in non-inflamed simulated epithelium (Fig. 2A, lined bars). When infant formula and *Lf* were combined, mucus production was augmented independently of protein form and inflammation (0.53–0.67 μg mL^{-1}), compared to the same conditions without *Lf* (0.48–0.49 μg mL^{-1}; # $p < 0.05$). Under inflamed conditions, also formula matrices alone (w/o *Lf*) augmented mucus production compared to control (–) (* $p < 0.05$) albeit to a lesser extent.

Digested samples showed a similar trend, with a similar increase of mucus production by both milk matrices and eHF + *Lf* (0.665 ± 0.027 μg mL^{-1}, $p < 0.05$), especially in LPS-condition and compared to control (+) (0.625 ± 0.014 mg ml^{-1}) (ESI Table S7†).

On mRNA expression level in non-LPS conditions, the expression of MUC2 was increased by eHF or eHF + *Lf* when compared to control (–) (* $p < 0.05$), whereas iPF only increased MUC2 when combined with *Lf* (* $p < 0.05$) (Fig. 2B). *Lf* alone did not significantly change MUC2 expression (Fig. 2B).

Overall, the MUC2 expression in eHF, eHF + *Lf* and eHF + *Lf* + LPS was significantly higher than in iPF, iPF + *Lf* and iPF + *Lf* + LPS, respectively ($ $p < 0.05$). Cells exposed to digested samples, independently of infant formula or *Lf* combination, showed a significant increase in MUC2 mRNA expression (1.7–2.6 fold-change log 2 units; ESI Table S8†), and again with stronger effects in hydrolysate based matrices.

Improvement of epithelial barrier function by *L. fermentum* CECT 5716 and formula specific effects

The effect of the different formula matrices on restoration of the barrier function was determined *via* TEER and LY measurements. To test the barrier function of the intestinal triple co-culture model, SDS treatment was used to disrupt the intestinal epithelium. As expected, under inflammatory conditions (control (+)), paracellular diffusion of LY was significantly increased (6.8 ± 0.2%) and TEER (1022 ± 51 Ω cm^2) reduced, compared to non-inflammatory conditions (control (–) (LY = 5.1 ± 0.7%; TEER = 1214 ± 9 Ω cm^2; * $p < 0.05$)) (Fig. 3A and B), both being associated with a weakened barrier. Exposition to eHF or iPF, significantly reduced LY transport (eHF = 4.6–4.8%; iPF = 5–5.2%; # $p < 0.05$) and increased TEER values (eHF = 1350–1405 Ω cm^2; iPF = 304–311 Ω cm^2; # $p < 0.05$, * $p < 0.05$), independently of LPS co-exposure thus improving epithelial barrier integrity (Fig. 3A and B). *Lf* treatment also improved the epithelial barrier and intestinal function (# $p < 0.05$) (Fig. 3A and B).

Digested samples of eHF and iPF, independently of *Lf*, reduced the percentage of LY transport compared to control

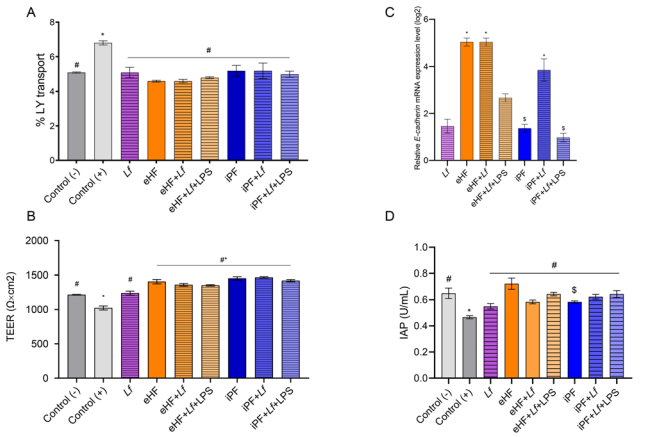

Fig. 3 Effect of infant formula matrices and postbiotic *L. fermentum* CECT 5716 on epithelial barrier function. (A) Bars represent the % of LY transport to the basolateral compartment (mean ± standard deviation; n = 6). (B) Bars represent the TEER values (mean ± standard deviation; n = 6) [ohms (Ω) cm^2]. Significant differences between control (–) and treatments are marked with an asterisk (* $p < 0.05$). Significant differences between control (+) and treatments are marked with a dash symbol (# $p < 0.05$). (C) Expression ratio (2 log units) (mean ± standard error of the mean; n = 6) of *E-cadherin* mRNA expression relative to control (–). Significant differences ($p < 0.05$) compared to control (–) are marked with an asterisk (*). Significant differences between different matrices (*i.e.*, iPF *vs.* eHF; iPF + *Lf vs.* eHF + *Lf*; iPF + *Lf* + LPS *vs.* eHF + *Lf* + LPS) are marked with a dollar symbol ($ $p < 0.05$). (D) Intestinal alkaline phosphatase (IAP) activity (mean ± standard deviation; n = 6). Significant differences between control (–) and treatments are marked with an asterisk (* $p < 0.05$). Significant differences between control (+) and treatments are marked with a dash symbol (# $p < 0.05$). Significant differences between different matrices (*i.e.*, iPF *vs.* eHF; iPF + *Lf vs.* eHF + *Lf*; iPF + *Lf* + LPS *vs.* eHF + *Lf* + LPS) are marked with a dollar symbol ($ $p < 0.05$).

(+) ($p < 0.05$), but only eHF + *Lf* significantly increased TEER values (ESI Table S10†).

Expression of *E-cadherin*, a main protein in the adherens junction, was strongly increased by native eHF, eHF + *Lf* and iPF + *Lf* (* $p < 0.05$) (Fig. 3C). In contrast, *Lf* alone, or iPF alone did not increase *E-cadherin* expression, which was significantly lower compared to eHF ($ $p < 0.05$). Despite the reinforcing effect of *Lf* in iPF (iPF + *Lf*), *E-cadherin* expression did not reach the high *E-cadherin* levels of eHF + *Lf*, showing a matrix effect of hydrolyzed *vs.* intact protein based formula.

A similar trend was observed in digested samples. Digested eHF + *Lf* increased *E-cadherin* expression (6.1 ± 0.4 log 2 fold change, $p < 0.05$) compared to control (–), and a similar, but less evident increase was observed for digested iPF (1.8 ± 0.6 log 2 fold-change, $p < 0.05$) (ESI Table S11†). Contrarily, digested samples from eHF and iPF + *Lf* decreased ($p > 0.05$) *E-cadherin* expression.

Further, IAP was assessed as a barrier supportive enterocyte differentiation marker and anti-inflammatory molecule. LPS-treatment (control (+)) reduced IAP (0.47 ± 0.2 U mL^{-1}) compared to control (–) (0.65 ± 0.07 U mL^{-1}) (* $p < 0.05$). *Lf*, native or digested infant formula matrices and combinations thereof, recovered IAP levels, reaching values similar to non-inflamed conditions (# $p < 0.05$) (Fig. 3D, ESI Table S12†).

L. fermentum CECT 5716 and formula matrices can attenuate the proinflammatory environment in a simulated intestinal epithelium

Generation of proinflammatory conditions by exposure of the simulated intestinal epithelium to LPS challenge [control (+)] reduced TGFβ protein levels below 50% compared to control (–) conditions (* $p < 0.05$) (Fig. 4A; ESI Table S13†). Exposure to *Lf*, eHF or iPF did not recover TFGβ levels (* $p < 0.05$). Contrarily, the combination of eHF + *Lf* and iPF + *Lf* significantly induced TGFβ production in inflamed and physiologic conditions (* $p < 0.05$; # $p < 0.05$) (Fig. 4A). *Lf* had a higher reinforcing effect on TGFβ levels in eHF matrix compared to iPF matrix ($ $p < 0.05$) (Fig. 4A). In digested samples, TGFβ production was increased by eHF + *Lf* and iPF + *Lf* to a similar extent ($p < 0.05$; ESI Tables S14 and S15†).

IL10 secretion was not induced by *Lf* and/or infant formula matrices in physiologic conditions (without LPS). However, when cells were under LPS-proinflammatory trigger, the synergistic combination of *Lf* with eHF or iPF increased IL10 levels (622–1082%) compared to respective controls (* $p < 0.05$, # $p < 0.05$), with the highest increase observed in the synbiotic intact protein matrix (iPF + *Lf* + LPS) exposed cells ($ p <0.05)

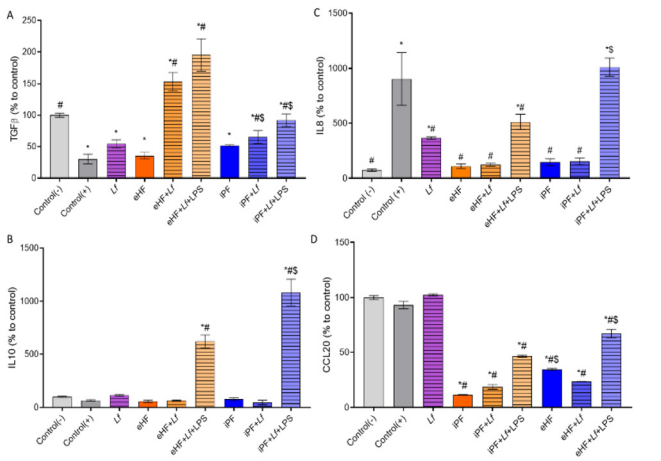

Fig. 4 Effect of infant formula matrices and postbiotic *L. fermentum* CECT 5716 in the pro-inflammatory environment of a simulated intestinal epithelium. (A) Percentage (mean ± standard deviation; n = 6) of TGF-β (A) and IL10 (B) protein levels in the basolateral compartment of the transwell system, compared to control (–). Percentage (mean ± standard deviation; n = 6) of IL8 (C) and CCL20 (D) protein levels in the apical compartment of the transwell system, compared to control (–). Significant differences ($p < 0.05$) between control (–) condition and different treatments are marked with an asterisk (* $p < 0.05$). Significant differences between control (+) condition (LPS) and different treatments are marked with a hash symbol (# $p < 0.05$). Significant differences between different matrices (*i.e.*, iPF *vs.* eHF; iPF + *Lf vs.* eHF + *Lf*; iPF + *Lf* + LPS *vs.* eHF + *Lf* + LPS) are marked with a dollar symbol ($ $p < 0.05$).

(Fig. 4B). In digested samples, IL10 was increased by eHF + *Lf*, iPF and iPF + *Lf* when compared to control (+), with the highest value observed for eHF + *Lf* (21.2 ± 6.5 pg ml^{-1}) (ESI Tables S14 and S15†).

IL8 secretion was significantly induced in all conditions with LPS trigger (* $p < 0.05$), independently of the infant formula matrix (Fig. 4C). Combination of eHF + *Lf* + LPS showed IL8 levels lower than control (+) (# $p < 0.05$), suggesting mitigation of the pro-inflammatory environment. This effect was not observed in iPF + *Lf* + LPS which was also significantly higher compared to eHF + *Lf* + LPS ($ $p < 0.05$). While *Lf* alone increased IL8 secretion above control (−) (* $p < 0.05$), when co-administered with infant formula, no increase was observed. In digested samples, milk matrices combined or not with *Lf* reduced IL8 levels compared to control (+) ($p < 0.05$; ESI Tables S14 and S15†).

CCL20 was significantly reduced by all infant formula matrices (* $p < 0.05$; # $p < 0.05$). *Lf* or LPS (control (+)) did not significantly affect CCL20 production, whereas milk matrices alone (eHF, iPF) or in combination with *Lf* in non-inflamed (eHF + *Lf*, iPF + *Lf*) and inflamed condition (eHF + *Lf* + LPS, iPF + *Lf* + LPS) reduced CCL20 compared to control (−), control (+) or *Lf* values (* $p < 0.05$; # $p < 0.05$). In some of the treatments, a matrix effect on CCL20 expression levels was observed ($ $p < 0.05$). In digested samples, only milk matrices without *Lf* reduced CCL20 levels, compared to control (+) ($p < 0.5$; ESI Tables S14 and S15†).

In contrast to protein levels, expression of *TGFβ* mRNA was significantly increased in all conditions (ESI Fig. S1†) (* $p < 0.05$), including infant formula matrices (eHF and iPF) and their synergistic combination with *Lf*, independently of LPS. *IL*10 mRNA expression was only significantly increased by eHF + *Lf* under proinflammatory conditions (LPS; * $p < 0.05$) (ESI Fig. S1†). Analysis of *TGFβ* and *IL*10 mRNA expression was not performed on cells exposed to digested samples.

Regulation of TLR expression and reduced TLR4-NFkB inflammatory activation by extensively hydrolyzed infant formula

The modulation of TLR4 signalling was analysed using a human TLR4-expressing HEK 293 reporter cell line. TLR4 signalling was significantly reduced by all the tested treatments, when compared to the LPS-proinflammatory condition (control (+), # $p > 0.05$) (Fig. 5). TLR4 activity was similar to control (−) (cell culture media) in *Lf*, eHF and eHF + *Lf* conditions, whereas iPF, iPF + *Lf* and both matrices in combination with *Lf* and LPS (eHF + *Lf* + LPS, iPF + *Lf* + LPS) induced TLR4 activation above control (−) (* $p < 0.05$). In non-inflamed conditions, the attenuating effect on TLR4 activity was more pronounced in the eHF matrix compared to iPF matrix ($ $p < 0.05$). In addition, mRNA expression of TLR2/4/9 and TOLLIP was analysed (ESI Fig. S2A–D†). Analysis of TLR4 was not performed on cells exposed to digested samples.

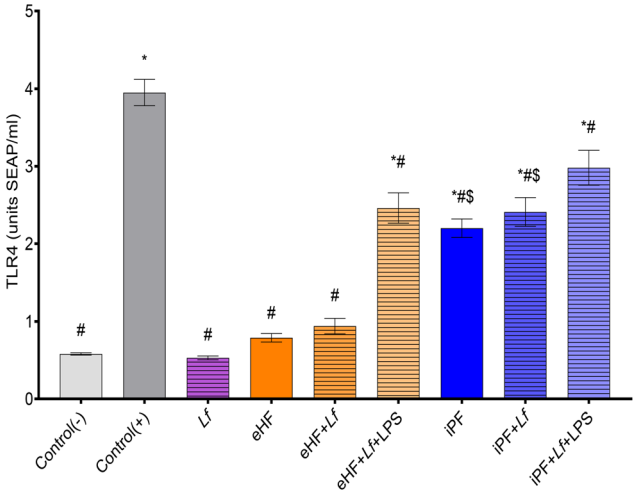

Fig. 5 Effect of infant formula matrices and postbiotic *L. fermentum* CECT 5716 in TLR4 signalling pathway. Bars represent the percentage (mean ± standard deviation) of SEAP activity in human TLR4-expressing HEK 293 cells (units mL^{-1}) in presence of different infant formula matrices without or with *Lf* and/or LPS (10 ng mL^{-1}). Significant differences between control (−) and different treatments are marked with an asterisk (* $p < 0.05$). Significant differences between control (+) and different treatments are marked with a dash symbol (# $p < 0.05$). Significant differences between different matrices (*i.e.*, iPF *vs.* eHF; iPF + *Lf vs.* eHF + *Lf*; iPF + *Lf* + LPS *vs.* eHF + *Lf* + LPS) are marked with a dollar symbol ($ $p < 0.05$).

Discussion

Our results report the impact of two different types of infant formula matrices and a probiotic strain *L. fermentum* CECT 5716 in viable or postbiotic form on intestinal homeostasis. While both formula matrices contain prebiotics GOS, the intact protein formula (iPF) and extensively hydrolyzed formula (eHF) differed in the form of protein present in the formula. We used an *in vitro* model of the gut to assess the effect of different infant formula matrices (eHF and iPF) combined or not with heat-inactivated *L. fermentum* CECT 5716 (*Lf*). We tested the different combinations *in vitro*, on the intestinal barrier, maturation, mucus and cytokine production, in the presence and absence of inflammatory triggers, and in native and digested conditions (Fig. 6).

We observed that iPF and eHF modulate adhesion-capability of viable *Lf*, compared to cell culture media, potentially due to interaction of *Lf* with proteins, sugars and fats present in the formula, as previously reported for rennet casein and bovine serum albumin on *Lacticaseibacillus casei* FMP and *Lactobacillus gasseri* adhesion to Caco2/HT29MTX cells.[35] Here, *Lf* adhesion was higher in combination with hydrolysate based infant formula than in intact protein based infant formula. This effect might be either due to changes in the surface proteins[36] or the better bioavailability of the hydrolyzed protein in eHF resulting in an increased probiotic growth and biomass,[37] resembling a prebiotic potential of the hydrolysate.[36–38]

Early infancy is a sensitive period for immune programming and gut homeostasis. Here, breastfeeding practices play a pivotal role due to the complex human milk composition including oligosaccharides, orally ingested antigens, microorganisms and other bioactive molecules.[39] The weaning period has been considered a physiological, low-grade inflammation process, with a complex development involving dietary antigen exposure due to diet changes and food introductions, gut barrier functioning, microbial shifts and orchestration of innate and adaptive immune responses.[40] Disturbances during the weaning process can lead to increased susceptibility to allergy.[41,42] Therefore, the present work addresses in a cell culture model the capacity of different forms of infant-formula matrices and probiotic supplementation on intestinal barrier and immune parameters in simulated pro-inflammatory conditions.

Infant formula based on partially or extensively hydrolyzed protein including or not probiotics and prebiotics, have been used for many years for infants at risk for allergy development who cannot be exclusively breastfed.[43–46]

Despite the evidence available, there is still uncertainty regarding the actual efficacy of a particular formula to reduce the risk of developing allergy in infants and lack of mechanistic information behind the observed effects.[46] As each protein hydrolysate has different properties (*e.g.* degree of hydrolysis), comprehensive research for each specific product is needed.

The rationale for selecting *Lf* was based on the characteristics that this strain was originally isolated from human milk and has been reported to promote beneficial effects *in vitro*, *in vivo*, and in human studies.[12,14,21]

Additionally, adhesion capabilities have been linked to live bacteria although inactivated bacteria can also adhere and interact with the host cells and mucus.[47]

Besides infant formula matrix affecting adhesion of the probiotic, we observed a significant increase in mucus production and *MUC2* mRNA expression, especially induced by *Lf*. Mucus production and *MUC2* mRNA expression were sensitive to pro-inflammatory LPS trigger, suggesting a differential response of goblet cells to infant formula matrices and/or *Lf* depending on intestinal environment. Under pro-inflammatory LPS-challenge, mucus production was significantly increased by infant formula, and further enhanced by *Lf* co-exposure. *MUC2* mRNA expression, however, was remarkably up regulated by eHF and eHF + *Lf*, and in a lesser extent by iPF + *Lf*. Multiple bacterial strains from lactic acid bacteria have been shown to modulate gene expression of MUC2 and other mucus-related proteins *in vitro.*[41] It has been reported that higher mucus production might protect against allergic sensitization and diet would play a key role in this mucus mediated effect.[47,48] When the mucus layer is reduced, exposure of epithelial cells to environmental stimuli like food antigens, commensal or pathogenic microorganisms is increased. Mucus production is immature during the neonatal period and the pivotal contact with microbes and other triggers from breastmilk, stimulates goblet cell maturation and mucus production.[49] Interestingly, *Lf* combined with the herein investigated infant formulas (eHF + *Lf*, iPF + *Lf*) or eHF alone can promote mucus production thereby enhancing intestinal barrier function and protecting the intestinal epithelium of excessive contact with antigenic substances.

Adequate intestinal barrier function is required to maintain intestinal homeostasis.[50] Moreover, it has been reported that infants with early allergic symptoms have increased gut permeability for proteins in comparison to non-allergic infants.[51] Probiotics can help to restore the barrier integrity as shown for *L. fermentum* strain MTCC-5898, which restored *E. coli*-damaged intestinal barrier integrity.[52] In agreement, we found that heat-inactivated *Lf* recovered epithelial barrier function after SDS/LPS treatment/challenge. Intestinal barrier enhancement, reported as TEER increase and LY transport decrease, was also observed for infant formula conditions, with no further improvement by *Lf*. To elucidate the mechanism behind, we analysed *E-cadherin* mRNA expression. Remarkably, we observed an upregulation of *E-cadherin* by eHF and eHF + *Lf*. This result suggests a main role of eHF in increasing *E-cadherin* mRNA expression, and further research is required to determine the specific components driving this effect. *E-cadherin* is the core component of the epithelial adherens junction protein, with a critical role in nucleating formation of adherens junctions, tight junctions and desmosomes, affecting structural tissue integrity and intestinal homeostasis.[53] *E-Cadherin* has also been reported to participate in intracellular signalling and cell behaviour control, with extensive cross-talk with the epithelial microenvironment, including the microbiota.[54] Fine-tuned regulation of adherens and tight junctions is required to act as a physical barrier against pathogen invasion and transfer of antigenic substances to the lamina propria.[49,54] In different murine models of intestinal inflammation, probiotic treatments with different lactobacilli improved epithelial barrier *in vivo*, through upregulation of *E-cadherin* or other cell-junction proteins.[50,51] These results could be confirmed in the present study with heat-inactivated (postbiotic) *Lf.* Expression of *E-cadherin* was not affected by iPF alone, while combination with *Lf* (iPF + *Lf*) induced expression, which suggests a synergistic effect on improving intestinal barrier control.

The multilayered control of intestinal homeostasis also involves soluble factors, such as intestinal alkaline phosphatase (IAP) or cytokines. IAP is physiologically produced by enterocytes and plays a vital role in detoxifying Gram-negative-derived LPS and avoiding the transmigration of bacteria across the epithelium. Thereby it regulates intestinal tolerance and inflammation.[55–57] Furthermore, the balance between pro- and anti-inflammatory signals is required at specific windows of susceptibility to promote adequate immune priming, adaptive training and tolerance.[1,58,59] Our study showed that IAP secretion is reduced under a pro-inflammatory challenge (*i.e.*, LPS), and recovered by infant formula, heat-inactivated *Lf* or combinations thereof, suggesting a protective role of *Lf* or infant formula towards recovery of intestinal homeostasis. Recently, a higher abundance of peptides associated with anti-inflammatory effects in the here tested formula was shown.[60]

Alongside, TGFβ and IL10 were increased by *Lf* under pro-inflammatory conditions, whereas IL8 was reduced, especially in combination with eHF, showing the induction/support of regulatory mechanism during pro-inflammatory simulations. In agreement with our findings, orally administered probiotics (*i.e.* bifidobacteria or lactobacilli) reduced the allergic-type immune response by supporting the lymphocyte T helper cell response, increasing IL10 and TGF-β production and improving immunological tolerance.[61–64] Furthermore, low abundance in gut lactobacilli was associated with higher levels of atopy-related markers.[65] Panahipour *et al.* reported that a hypoallergenic infant formula lacked *in vitro* TGFβ activity and had lower anti-inflammatory activity compared to a standard formula.[66] Remarkably, in our study we observed that eHF alone is capable to induce TGFβ protein expression *in vitro* (at levels comparable to control (+)) and that the combination of eHF + *Lf* recovers and even exceeds TFGβ production, especially under pro-inflammatory conditions. This clearly illustrates the benefit of combining eHF and *Lf*. Different cell types, pro-inflammatory stimuli and infant formula matrices may cause the differences observed in our study. Björkander *et al.*, correlated the presence of certain lactobacilli species in the infant gut with allergy–related parameters in the peripheral immune system, including CCL20 cytokines, suggesting a role of specific lactobacilli on allergy protection.[65] In our system, CCL20 levels were not responsive to LPS, as observed by similar levels of this cytokine in control (−) and control (+). Sierro *et al.*, described that the expression of the CCL20 gene in human intestinal epithelial cell lines Caco-2 and T-84 was up-regulated by pathogenic bacteria, but not by commensal intestinal bacteria, whereas CCL20 activation was dependent on flagellin and not lipopolysaccharide.[67] These findings could partially explain the unresponsiveness of Caco-2 cells against LPS.

In this study, infant formula matrices and *Lf* modulated TLR4 signaling using a human TLR4-expressing HEK 293 reporter cell line. As expected, heat-inactivated *Lf* or infant formulas did not activate the TLR4 signaling pathway to control (+) levels. In contrast, under pro-inflammatory conditions, the LPS-induced TLR4 activation was attenuated by eHF + *Lf* and iPF + *Lf*, suggesting a potential anti-inflammatory mechanism *in vitro*.[68]

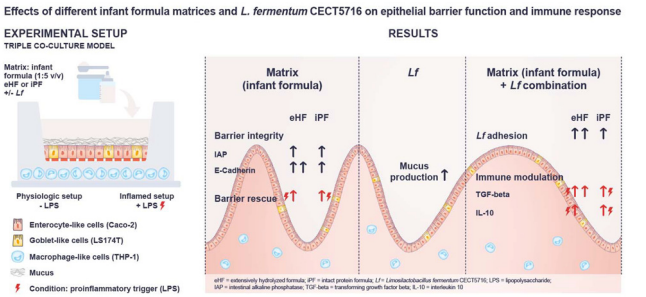

Fig. 6 Graphical Abstract – **Effect of different infant formula matrices and *L. fermentum* CECT 5716 on epithelial barrier function and immune response.** A triple co-culture transwell setup containing enterocyte-like cells (Caco-2), goblet-like cells (LS174T) and macrophage-like cells (THP-1) was used to simulate a model of gut inflammation (+LPS) and homeostasis (−LPS). The effect of extensively hydrolyzed infant formula (eHF) compared to cow's milk based standard infant formula (iPF) combined or not with heat-inactivated *Limosilactobacillus fermentum* CECT 5716 (*Lf*) on the intestinal barrier integrity and cytokine production was analysed. *Lf* alone was able to induce mucus secretion and thus strengthens the epithelial barrier. In the physiologic setup (−LPS), eHF had stronger effects on barrier integrity (*E-cadherin* expression) and promoted *Lf* adhesion to the simulated epithelium. Under LPS-inflammatory trigger, iPF had positive effects on immune modulation (regulatory TGFβ, IL-10) and barrier integrity and −rescue, while these effects were even exceeded in eHF matrix (eHF ± *LF* ± LPS).

Limitations

With regard to the setup of this *in vitro* study we acknowledge that the use of cell lines, whether SEAP-reporter lines or immortalized cells, is limited in describing the concerted action of immune, barrier and microbial cells. However, similar triple co-culture systems have been previously reported and have been able to give valuable insight to the interaction of microbial and immune cells.[21,22] Of note, the use of infant formula directly added on the cell systems cannot fully represent the complex digestive processes altering the formula matrices. To reduce this limitation, digested formulas were also used in some but not all of the setups. This offers a proof of concept for further studies including more complex approaches. The strain *Lf* could not be used consistently as a pro- or postbiotic strain, as different properties of the strain were required depending on the experimental setup. *E.g.* for the adhesion assay the strain must be alive, while experimental setups with longer incubation periods in cell culture provide more reliable results with a non-replicating post-biotic strain.[69] Further, consistent results were not obtained for all parameters analysed. For example, the mRNA and protein expression of MUC2 differed. However, discrepancy between the abundance of cognate protein and mRNA molecules is frequently observed in mammalian and yeast cells. The processes of transcription, translation, and degradation of mRNA molecules and dissociated time-frame scales for mRNA and protein production can cause discrepancies between both markers. This is already described in literature and widely accepted by the scientific community.[70,71]

Conclusions

In conclusion, we observed that eHF, especially in combination with *Lf*, significantly preserved the intestinal barrier by inducing mucus production as well as *MUC2* and *E-cadherin* mRNA expression. Furthermore, immunomodulation of the LPS-induced local inflammatory response was achieved by increasing IL10 and TGFβ levels and decreasing IL8 levels and TLR4 signalling. Therefore, this study provides information on potential mechanisms influencing intestinal homeostasis and immune response after ingestion of infant formula based on

intact proteins or extensively hydrolyzed proteins, which is crucial for infants who cannot be breastfed. Future studies will need to confirm these findings in an *in vivo* scenario and extend our knowledge on key elements involved in allergy development or risk reduction, such as intestinal T cell and neutrophil responses.

Abbreviations

eHF	Extensively hydrolyzed formula
iPF	Intact protein formula
Lf	*Limosilactobacillus fermentum* CECT 5716
HM	Human milk
GOS	Galactooligosaccarides
ECACC	European collection of authenticated cell cultures
MRS	Man, Rogosa and Sharpe agar
DMEM	Dulbecco's modified Eagle's medium
NAC	*N*-acetyl-L-cysteine
HBSS	Hanks balanced salt solution
PBS	Phosphate buffered saline
LY	Lucifer Yellow
TEER	Transepithelial electrical resistance
LPS	Lipopolysaccharide
SDS	Sodium dodecyl sulfate
RT-PCR	Real-Time polymerase chain reaction
MUC2	Mucin 2
IAP	Intestinal alkaline phosphatase
TGFβ	Transforming growth factor beta
IL	Interleukin
TLR	Toll like receptor
SEAP	Secreted embryonic alkaline phosphatase
PNPP	*p*-Nitrophenyl phosphate disodium salt
PMA	12-*O*-Tetradecanoylphorbol-13-acetate
iFBS	Inactivated fetal bovine serum
AFU	Arbitrary fluorescent units
df	Dilution factor
VF	Final volume of the reaction
VE	Sample volume
NFKβ	Nuclear factor kappa-light-chain-enhancer of activated B cells

Author contributions

MAC, MS: conceptualization, writing – original draft, methodology, investigation, validation, visualization, and data interpretation; LO: investigation, validation; MCC, EMH, MS: supervision, conceptualization, writing – review and editing, visualization, and interpretation.

Data availability

The data supporting this article have been included as part of the ESI.†

Conflicts of interest

MCC has been compensated for speaking engagement in meetings sponsored by HiPP GmbH & Co. Vertrieb KG and also, for other companies Danone, Nutricia, Nestlé Nutrition Institute and Mead Johnson-Reckitt. MAC has been compensated for speaking engagement at one meeting sponsored by HiPP GmbH & Co. Vertrieb KG. EMH and MS are employees of HiPP GmbH & Co. Vertrieb KG, 85276 Pfaffenhofen, Germany.

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