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Colostrum whey down-regulates the expression of early and late inflammatory response genes induced by *Escherichia coli* and *Salmonella enterica* Typhimurium components in intestinal epithelial cells

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Abstract

Pathogenic invasion by *Escherichia coli* and *Salmonellae* remains a constant threat to the integrity of the intestinal epithelium and can rapidly induce inflammatory responses. At birth, colostrum consumption exerts numerous beneficial effects on the properties of intestinal epithelial cells and protects the gastrointestinal tract of newborns from pathogenic invasion. The present study aimed to investigate the effect of colostrum on the early and late inflammatory responses induced by pathogens. The short-term (2 h) and long-term (24 h) effects of exposure to heat-killed (HK) *E. coli* and *Salmonella enterica* Typhimurium on gene expression in the porcine intestinal epithelial cell (IPEC-J2) model were first evaluated by microarray and quantitative PCR analyses. Luciferase assays were performed using a NF-κB-luc reporter construct to investigate the effect of colostrum whey treatment on the activation of NF-κB induced by HK bacteria. Luciferase assays were also performed using NF-κB-luc, IL-8-luc and IL-6-luc reporter constructs in human colon adenocarcinoma Caco-2/15 cells exposed to dose–response stimulations with HK bacteria and colostrum whey. Bovine colostrum whey treatment decreased the expression of early and late inflammatory genes induced by HK bacteria in IPEC-J2, as well as the transcriptional activation of NF-κB-luc induced by HK bacteria. Unlike that with colostrum whey, treatment with other milk fractions failed to decrease the activation of NF-κB-luc induced by HK bacteria. Lastly, the reduction of the HK bacteria-induced activation of NF-κB-luc, IL-8-luc and IL-6-luc by colostrum whey was dose dependent. The results of the present study indicate that bovine colostrum may protect and preserve the integrity of the intestinal mucosal barrier in the host by controlling the expression levels of early and late inflammatory genes following invasion by enteric pathogens.

Key words: Transcription regulation: *Escherichia coli*: *Salmonellae*: Intestinal epithelial cells: Inflammatory responses

Infections caused by *Escherichia coli* and *Salmonellae* can significantly affect animal and human health as well as food safety. Although these bacteria are fundamentally very different, there are some similarities between their pathogenesis. For instance, infections caused by enterotoxigenic *E. coli* (ETEC) and *Salmonella enterica* serovar Typhimurium usually occur in the epithelium of the gastrointestinal tract and can lead to cramps, diarrhoea and vomiting[1–4]. In addition, both pathogens have the capacity to stimulate innate immune responses and inflammatory cytokine production[5–10]. Indeed, the expression of *IL8* is rapidly induced in porcine intestinal epithelial cells (IPEC-J2) after exposure to ETEC or *Salmonella* challenges as well as by the toxins produced by these bacteria[11–13].

In domestic animals, infections caused by *E. coli* and *Salmonella* lead to reduced feed intake and growth performance and can ultimately cause death, thereby generating serious economic losses due to impaired animal productivity. Moreover, contamination by infected animals during slaughter can have potentially devastating consequences for the food industry and ultimately for consumers. To prevent infections as well as to promote animal growth, subclinical doses of antibiotics can be added to animal feed, such as for swine and poultry. Although this practice is still accepted in North America, it has been severely criticised and banished in Europe, because of the threat it generates regarding possible emergence of antimicrobial resistance in the environment and potential negative effects on public health. Therefore, many efforts are being made to find novel approaches, such as the use of harmless natural products as feed additives, to prevent enteric infections caused by ETEC, *Salmonella* and other pathogens. Although numerous additives and ingredients,

Abbreviations: *CCL*, chemokine (C-C motif) ligand; CFU, colony-forming unit; ETEC, enterotoxigenic *Escherichia coli*; HK, heat killed; IPEC-J2, porcine intestinal epithelial cells; q-PCR, quantitative PCR.

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namely probiotics and prebiotics, have the potential to replace in-feed antibiotics, their effectiveness is often controversial and remains to be demonstrated[14]. Among other complementary alternatives, bovine milk and colostrum, as well as other milk products such as cheese whey and milk peptides, have been identified for their potential to modulate intestinal defence functions and microbiota establishment[15–19].

Colostrum, the first milk produced after birth, is rich in Ig and growth factors as well as immunoregulatory and antimicrobial factors[20]. This combination of bioactive ingredients generates optimal conditions in the gastrointestinal tract of newborns to promote health and resistance to external threats. Indeed, the high content of Ig and various factors involved in the development and regulation of the immune system contributes to the provision of passive immunity to the offspring after birth and protection against bacterial and viral infections[21,22].

Interestingly, most of the bioactive components of colostrum are found in the soluble whey portion after the removal of casein. Indeed, concentrated Ig, lactoferrin, growth factors, and lactoperoxidase as well as most of the immune cell factors are recovered in the whey fraction following isoelectric precipitation[23,24]. Therefore, the use of bovine colostrum whey as a feed additive to prevent enteric infections and to promote growth appears to be an interesting approach to improve intestinal health. In fact, studies have shown that bovine colostrum concentrates attenuate the symptoms of acute haemorrhagic diarrhoea in children infected with enterohaemorrhagic *E. coli* and that enterohaemorrhagic *E. coli* haemolysin can be neutralised by Ig isolated from bovine colostrum[25,26]. Bovine colostrum exerts protective effects in mice infected with *E. coli* through the inhibition of bacterial attachment to the intestinal mucous membrane and through the prevention of both bacterial colonisation and growth in the intestinal tract[27]. Piglets fed a weanling diet supplemented with bovine colostrum exhibit an improvement in growth performance and a decrease in diarrhoea episodes when compared with control piglets[28], while a formula containing bovine colostrum whey is better than others at improving intestinal defence function and protecting against necrotising enterocolitis[29]. Moreover, different products of bovine whey protein concentrates decrease the attachment of *Salmonella* Typhimurium and *E. coli* to Caco-2 cells[30] and bovine colostrum oligosaccharides reduce *Campylobacter jejuni* infections[31].

Although the beneficial effects of colostrum on intestinal health have been observed in various studies, the cellular mechanisms underlying these effects remain unknown. Considering the strong similarities and significant repercussions of ETEC and *Salmonella* infections for human and pig health, the components of these pathogens were used in the present study to stimulate the inflammatory responses of IPEC-J2 and human intestinal epithelial cells and to demonstrate the effect of bovine colostrum whey on the activation of the NF-κB transcription factor and the regulation of gene expression.

Materials and methods

Cell culture

Non-transformed IPEC-J2, derived from the jejunum of newborn piglets[32], were cultured in Dulbecco's modified Eagle's medium–Ham's F-12 (1:1) (Wisent) supplemented with 5% heat-inactivated fetal bovine serum (Wisent), insulin–transferrin–selenium (ITS premix; BD Biosciences), glutamine and 5 ng/ml epidermal growth factor (Wisent). For the induction of cell differentiation, confluent IPEC-J2 were grown in a fetal bovine serum-deprived medium supplemented with 10^{-7} M-dexamethasone (Sigma-Aldrich Canada) for 10 d. Caco-2/15 human colon adenocarcinoma cells[33] were cultured in Dulbecco's modified Eagle's medium high glucose (Invitrogen) supplemented with 10% fetal bovine serum, 25 mM-HEPES and glutaMAX (Invitrogen).

Preparation of colostrum whey and milk fractions

Bovine colostrum whey was prepared as described previously[34], by isoelectric precipitation (pH 4·6) of defatted colostrum, followed by centrifugal separation to remove the casein precipitate. Supernatants were freeze-dried. Cheese whey, obtained from a local cheese factory (L'Ancêtre), was skimmed, filtered (Tetra Pak MSF1) through a 1·4 μm membrane (Membralox™; Pall Corporation), concentrated by ultrafiltration through a 5 kDa membrane (Romicon; Koch Membrane Systems), freeze-dried and stored at −20°C. The final protein concentrations of colostrum whey and cheese whey were 68·1 and 71·8%, respectively. Both whey products were irradiated with a dose of 5 kGy using a Gammacell 220 irradiator unit (Atomic Energy of Canada Limited) and refrozen at −20°C. Bovine lactoferrin was obtained from DMV International. Bovine caseinomacropeptide was a kind gift from Dr G. Robitaille (Centre de recherche et de développement sur les aliments, Agriculture and Agri-Food Canada; St-Hyacinthe, QC, Canada) and was extracted as described previously[35]. For the *in cellulo* experiments, colostrum whey, milk whey, lactoferrin and caseinomacropeptide were diluted in Opti-MEM (Invitrogen).

Heat-killed bacteria

The ETEC F4 strain ECL8559 (0149:LT:STa:STb:East1:paa:hemβ:F4) and the *S. enterica* serovar Typhimurium DT104 # 4393 were, respectively, obtained from Dr J. M. Fairbrother[11] and Dr A. Letellier[36]. Bacteria were grown in Lysogeny broth (LB) medium to exponential phase and quantified on LB agar plates. Bacteria were precipitated by centrifugation, resuspended in Opti-MEM and killed by incubation for 1 h at 70°C. Stocks of *E. coli* and *Salmonella* Typhimurium (10^{12} colony-forming units (CFU)/ml) were kept frozen until use. Final diluted concentrations of 10^{11} CFU/ml, for the stimulation of IPEC-J2, and 10^{9}–10^{11} CFU/ml, for the stimulation of Caco-2/15 cells, were used.

Microarray analysis

Differentiated IPEC-J2 were stimulated with HK bacteria (10^{11} CFU/ml) for 2 and 24 h, with or without colostrum

whey (10 mg/ml). Total RNA were extracted using the Qiagen RNeasy Kit (Qiagen). All samples were submitted to RNA quality control with the Agilent 2100 Bioanalyzer (Agilent Technologies), using the RNA 6000 Nano Kit. Complementary DNA was synthesised using 25 ng of RNA, and hybridisation with the Agilent Porcine Gene Expression Microarrays was performed at the Microarray platform of the McGill University and Génome Québec Innovation Centre. Three independent experiments were performed for each condition. Data analysis, normalisation, and average difference and expression analyses for each feature on the chip were performed using FlexArray software version 1.6.1 (Génome Québec). Fold changes were determined by comparing gene expression in treated cells with that in control untreated cells and are reported in $\log_2$ scale. Genes with expression levels that were increased more than 2-fold ($\log_2 > 1$) after stimulation with HK bacteria were selected and analysed according to their Kyoto Encyclopedia of Genes and Genomes pathways and Gene Ontology classification, with Gene Ontology searches using 'Database for Annotation, Visualization, and Integrated Discovery, version 2008' (http://david.abcc.ncifcrf.gov/) and ToppGene Suite(http://toppgene.cchmc.org/).

Quantitative real-time RT-PCR

Differentiated IPEC-J2 were treated with HK bacteria for 2 and 24 h, with or without colostrum whey. RNA were prepared using the Qiagen RNeasy Kit (Qiagen). Complementary DNA were synthesised using oligo(dT) and SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative PCR (q-PCR) analysis was performed using the Power SYBR Green PCR Master Mix and the 7500 Fast Real-Time PCR System (Applied Biosystems and Life Technologies, Inc.). Primers used in the q-PCR analysis, designed with Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast), are listed in Table 1. Data were analysed by the $\Delta\Delta C_T$ method, using β-actin as the internal control. All data passed the normality and homogeneity of variance tests, and there were no significant differences in the quantity of β-actin RNA among all the RNA samples extracted. The identity and purity of the amplified product were checked through the melting curve analysis carried out at the end of amplification.

Luciferase assays

IPEC-J2 and Caco-2/15 cells were grown in twenty-four-well plates and were transiently transfected with the luciferase

Table 1. List of primers used in the quantitative PCR analysis corresponding to genes induced by heat-killed bacteria and regulated by colostrum whey, as determined by the microarray analysis

Gene symbol	RefSeq accession	Primer	Sequence (5′ → 3′)	Start	Stop
CCL2	NM_214214	Forward	TCGATGCAGCGGCTGATGAGCTA	182	204
		Reverse	CTGCACAGATCTCCTTGCCCGC	281	260
CCL4	NM_213779	Forward	TCCCTCCTGGTCCTGGTCGCT	53	73
		Reverse	TCCGCACGGTGTATGTGAAGCAG	152	130
CCL5	NM_001129946	Forward	GAAGGTCTCCACCGCTGCCC	63	82
		Reverse	ACAGCAGGGTGTGGTGTCCGA	162	142
CCL20	NM_001024589	Forward	GTCGGTGCTGCTGCTCTACCTC	76	97
		Reverse	GCTTCATTGGCGAGCTGCTGTG	207	186
CD40	NM_214194	Forward	CGCTGGGGATCCTGTTTGCCG	602	622
		Reverse	CGTCTCCACGGGATCCTGCCT	717	697
CHI3L1	NM_001001540	Forward	CTCCCCTGGGCGGAGAGACA	486	505
		Reverse	GCAGGAGCCGCTCTGTTCCA	586	567
CXCL2	NM_001001861	Forward	TTGTCTCAACCCCGCAGCCC	285	304
		Reverse	AGCAGTAGCCAGTAAGTTTCCTCCA	392	368
CXCL10	NM_001008691	Forward	TCAGCTGCAGCACCATGAACCA	55	76
		Reverse	TGCAGGTACAGCGAACAGTTCTGG	165	142
IL6	NM_214399	Forward	AGGGAAATGTCGAGGCTGTGC	473	493
		Reverse	AGGCCGGCATTTGTGGTGGG	587	568
IL8	NM_213867	Forward	CAGCCCGTGTCAACATGACTTCCA	70	93
		Reverse	GCACTGGCATCGAAGTTCTGCAC	191	169
IL19	XM_003130464	Forward	ACCAGGAGCTGGACCCCCAAC	278	298
		Reverse	TCCTGCCTGCAGTGGCATAGC	395	375
IL23A	NM_001130236	Forward	CGCTGGCCTGGACTGCACAT	134	153
		Reverse	ACAGCCATCCCCGCACTGGA	243	224
IRF1	NM_001097413	Forward	CGGATAGCGCTTGCTGCCGA	209	228
		Reverse	TCCAGATCAGCCCTGGGATTTGGT	313	290
ORM1	XM_001928727	Forward	AACCCGCTGTGCGCCAACTT	110	129
		Reverse	GGTTTCGGAAGGCCGAGCCG	209	190
SAA2	NM_001044552	Forward	TGTCAGCAGCCAGAGATGGGC	45	65
		Reverse	CGGGCATGGAAGTACTTGTCCGA	170	148
β-Actin	XM_003357928	Forward	CTCTTCCAGCCCTCCTTCCT	447	466
		Reverse	GCGTAGAGGTCCTTCCTGATGT	518	497

CCL, chemokine (C-C motif) ligand; *CD40*, CD40 molecule, TNF receptor superfamily member 5; *CHI3L1*, chitinase 3-like 1; *CXCL*, C-X-C motif chemokine ligand; *IL23A*, IL23, α-subunit p19; *IRF1*, interferon regulatory factor 1; *ORM1*, orosomucoid 1; *SAA2*, serum amyloid A2.

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reporter construct NF-κB-luc, IL-8-luc or IL-6-luc using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The reporter construct NF-κB-luc has multiple copies of a NF-κB response element that drives the transcription of the luciferase reporter gene, while the reporter constructs IL-8-luc and IL-6-luc have the promoter regions of *IL8* and *IL6* genes, respectively. Cells were treated with HK bacteria in combination with colostrum whey or other milk fractions for 6 h. Luciferase activity was measured and relative luciferase activity was determined by comparing treated samples with untreated control samples. All the experiments were carried out at least three times in duplicate.

Statistical analysis

Comparisons of quantitative data were made using a one-way ANOVA. When a significant difference was identified by ANOVA ($P<0{\cdot}05$), Tukey's test (*post hoc*) was carried out to identify differences among the groups ($P<0{\cdot}05$). The analysis was performed using the SigmaPlot software (version 12.5; Systat Software, Inc.). All data were found to comply with ANOVA assumptions. Results are expressed as means with their standard errors.

Results

Colostrum whey affects the expression of early and late genes induced by heat-killed bacteria

Among the genes induced by HK bacteria, sixty-two were known and characterised, while another seventy-one probes had no gene name associated with them, as revealed by the microarray analysis. To determine the cellular mechanisms triggered by bacterial components in IPEC-J2, known and

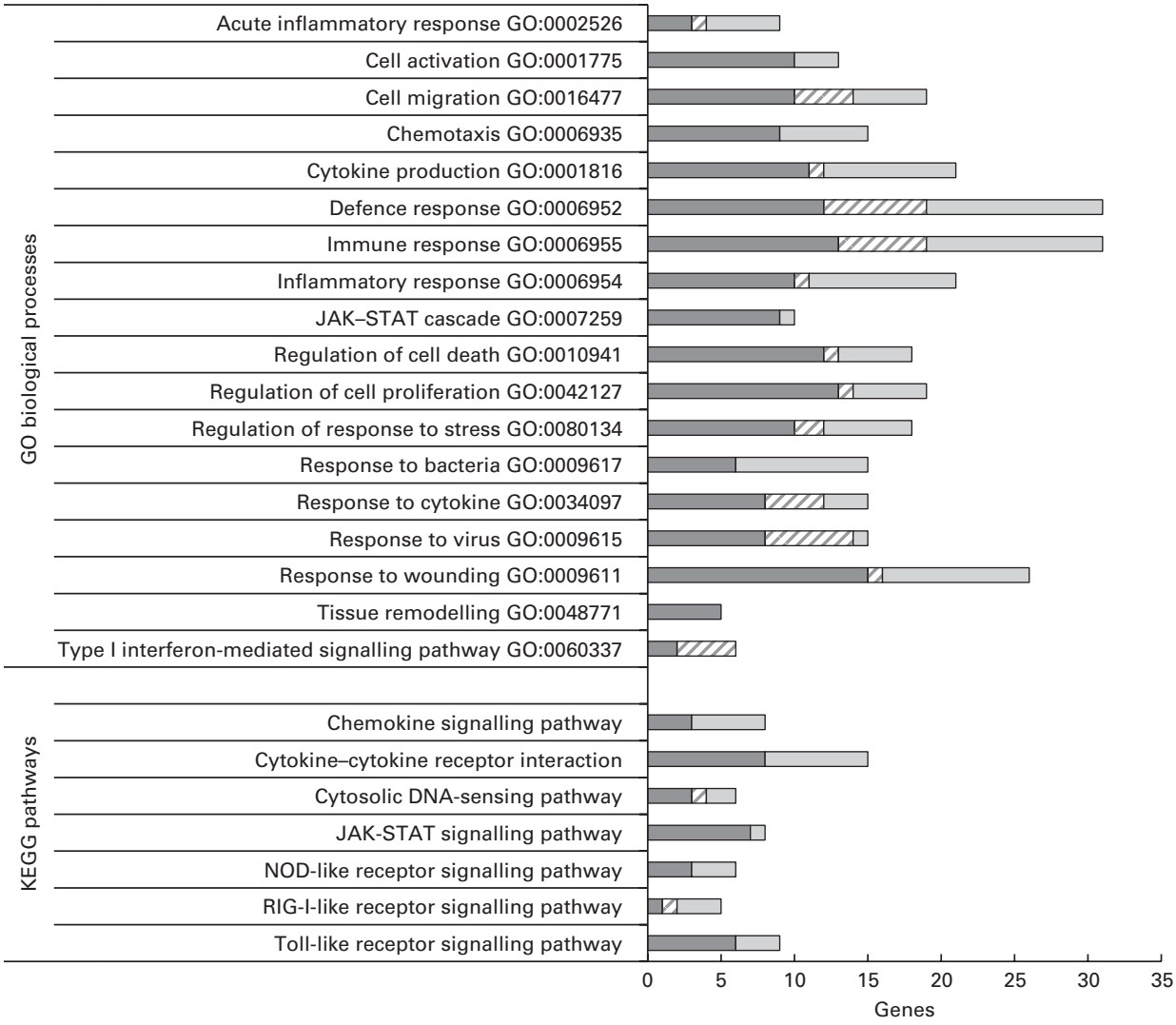

Fig. 1. Early and late biological processes and pathways in porcine intestinal epithelial cells affected by stimulation with heat-killed (HK) bacteria. Gene Ontology (GO) biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with genes significantly induced (fold induction >2; $P<0{\cdot}05$) after 2 h of stimulation with HK bacteria (early genes; ■), after 24 h of stimulation (late genes; ▨) or after both 2 and 24 h of stimulation (□). GO biological process searches were performed using ToppGene Suite (http://toppgene.cchmc.org/), while KEGG pathways were identified with 'Database for Annotation, Visualization, and Integrated Discovery, version 2008' (http://david.abcc.ncifcrf.gov/). JAK/STAT, Janus kinase/signal transducers and activators of transcription pathway; NOD, nucleotide-binding oligomerisation domain; RIG, retinoic acid-inducible gene.

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characterised genes were categorised according to their temporal expression patterns, based on their expression levels after 2 and 24 h of stimulation with HK bacteria. The expression of early genes was induced after 2 h, but their expression levels returned to control levels after 24 h (twenty-nine genes in total). On the other hand, the expression of fifteen late genes was not induced after 2 h, but their expression levels were elevated after 24 h. Lastly, the expression of eighteen genes was induced at both time points. These three groups of genes were classified according to the Gene Ontology biological processes and Kyoto Encyclopedia of Genes and Genomes pathways and the gene counts were compared (Fig. 1). Interestingly, classes including 'cell activation', 'tissue remodelling', 'JAK–STAT cascade' (Janus kinase/signal transducers and activators of transcription pathway), 'regulation of cell death' and 'cell proliferation' mainly comprised early genes. On the other hand, classes including 'type I interferon-mediated signalling pathway' and 'response to virus' comprised a significant number of late genes. The expression of genes involved in other classes of biological processes and Kyoto Encyclopedia of Genes and Genomes pathways related to immunity, inflammation, defence response, wounding and migration was induced early, late or at both time points by HK bacteria. The classification details of early genes (2 h), late genes (24 h) and genes induced at both time points are given in online supplementary Tables S1–S3, respectively.

The effect of colostrum whey treatment on the expression of genes after stimulation with HK bacteria was investigated. The HK bacteria induction of immune, defence and inflammatory response genes was mostly decreased by colostrum whey treatment. On the other hand, the induction by HK bacteria of genes involved in proliferation, wounding and migration processes was rather increased in colostrum whey-treated cells (Fig. 2). The effect of colostrum whey treatment on the expression of immune and inflammatory response genes after stimulation with HK bacteria is further summarised in Table 2, while the effect on that of other genes is summarised in Table 3.

The colostrum whey-dependent reduction of immune and inflammatory gene expression after stimulation with HK bacteria was further confirmed by the q-PCR analysis. Colostrum whey treatment decreased the expression of *IL8*, chemokine (C-C motif) ligand 5 (*CCL5*), serum amyloid A2 (*SAA2*), *IL6*, CD40 molecule, TNF receptor superfamily member 5 (*CD40*), *IL19*, chitinase 3-like 1 (*CHI3L1*) and *IL23A* after 2 h of stimulation with HK bacteria (Fig. 3(a)). The q-PCR analysis results also revealed that colostrum whey treatment decreased the expression of *CCL2* and increased the expression of chemokine (C-X-C motif) ligand 2 (*CXCL2*) after 2 h of stimulation with HK bacteria, in contrast to the microarray analysis results showing no significant differences in the expression of either gene after colostrum whey treatment. The q-PCR analysis results also failed to show a significant decrease in the expression of *CCL20* and interferon regulatory factor 1 (*IRF1*) after colostrum whey treatment, as revealed by the microarray analysis.

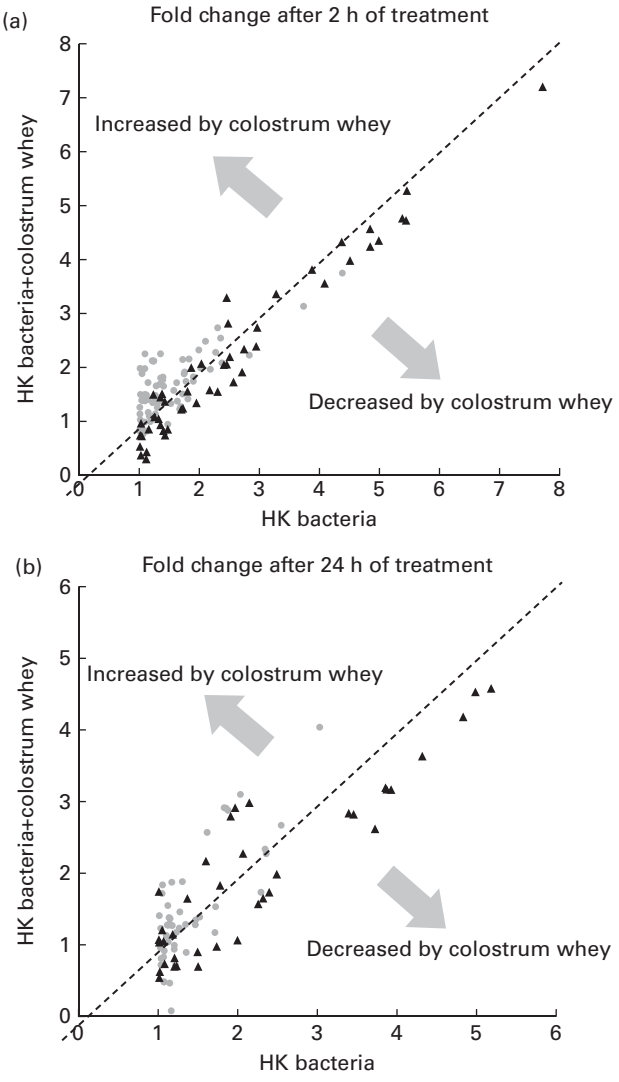

Fig. 2. Effect of colostrum whey on the expression of immune and inflammatory genes induced by heat-killed (HK) bacteria in porcine intestinal epithelial cells (IPEC-J2). Scatter plots of gene expression levels in IPEC-J2 after (a) 2 h and (b) 24 h of treatment with HK bacteria without (horizontal axis) or with (vertical axis) colostrum whey, as determined by the microarray analysis. Probes were selected according to the fold change after treatment with HK bacteria and their *P* value ($\log_2$ (fold change) >1; $P<0{\cdot}05$). Immune, defence and inflammatory response genes are indicated by ▲ and other genes by ●. Fold change was calculated by comparing the expression levels in treated cells with those in untreated (control) cells, and it is expressed in $\log_2$ scale.

The expression levels measured after 2 h of stimulation with HK bacteria were decreased after 24 h for most of the genes, except *SAA2*, *CHI3L1*, *CCL4*, orosomucoid 1 (*ORM1*) and *CXCL10*, the expression levels of which were either increased or remained unchanged (Fig. 3(b)). Colostrum whey treatment decreased the expression of *CCL5*, *SAA2*, *CHI3L1*, *ORM1* and *CXCL10*, but did not affect the expression of *IL8* and *IRF1*, confirming similar observations made during the microarray analysis.

Together, these results reveal that colostrum whey has marked immunoregulatory and anti-inflammatory properties,

Table 2. List of genes involved in immune and/or inflammatory responses exhibiting increased expression in porcine intestinal epithelial cells after 2 and 24 h of treatment with heat-killed bacteria (HK b), without or with colostrum whey (col), as determined by the microarray analysis‡

			Fold change after 2 h of treatment		Fold change after 24 h of treatment	
Probe name	Gene symbol	Gene name	HK b	HK b + col	HK b	HK b + col
A_72_P165601	*C3*	Complement component 3	1·73	1·25*	3·87	3·17*
A_72_P737664			1·70	1·22*	3·86	3·19*
A_72_P742730			1·43	0·75*	3·39	2·84*
A_72_P745528			1·48	0·85*	3·46	2·82*
A_72_P443993			0·93	0·42	2·39	1·73*
A_72_P035346	*CCL2*	Chemokine (C-C motif) ligand 2	1·38	1·51	0·11	0·38
A_72_P177441	*CCL20*	Chemokine (C-C motif) ligand 20	7·72	7·20*	2·49	1·99*
A_72_P223332	*CCL4*	Chemokine (C-C motif) ligand 4	1·80	1·56	0·15	0·10
A_72_P623643	*CCL5*	Chemokine (C-C motif) ligand 5	2·57	1·73*	0·86	0·46*
A_72_P115401			2·17	1·58*	0·67	0·42
A_72_P232617	*CD274*	CD274 molecule	1·95	1·34*	0·38	0·05
A_72_P088376	*CD40*	CD40 molecule, TNF receptor superfamily member 5	2·75	2·34*	0·41	0·12
A_72_P124966			2·71	1·91*	− 0·01	0·02
A_72_P544217			2·31	1·55*	0·18	0·14
A_72_P511075	*CHI3L1*	Chitinase 3-like 1 (cartilage glycoprotein-39)	2·41	2·05*	1·21	0·82*
A_72_P302864			2·51	2·20*	2·00	1·07*
A_72_P077726	*CSF2*	Colony-stimulating factor 2 (granulocyte-macrophage)	2·46	3·29*	1·01	1·74*
A_72_P165266	*CXCL10*	Chemokine (C-X-C motif) ligand 10	1·35	0·94*	2·32	1·65*
A_72_P509208			1·40	0·83*	2·26	1·57*
A_72_P146411	*CXCL2*	Chemokine (C-X-C motif) ligand 2	4·37	4·33	1·37	1·65
A_72_P732383			3·88	3·82	1·05	1·21
A_72_P223287	*DDX58*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	0·30	0·26	1·01	1·07
A_72_P286449	*IFIT1*	Interferon-induced protein tetratricopeptide repeats 1	0·08	− 0·17*	1·02	1·04
A_72_P402708	*IFITM1*	Interferon-induced transmembrane protein 1-like	0·19	− 0·21*	1·08	0·74*
A_72_P172311			0·14	0·21	1·50	0·90*
A_72_P232582	*IL12B*	Interleukin 12B	1·04	0·73	− 0·01	0·20
A_72_P443233	*IL19*	Interleukin 19	2·46	2·06*	0·87	0·45†
A_72_P444799	*IL1A*	Interleukin 1, alpha	3·28	3·36	2·15	2·98*
A_72_P165276			2·48	2·82*	1·60	2·17*
A_72_P414068	*IL23A*	Interleukin 23, alpha-subunit p19	2·95	2·39*	0·19	0·30
A_72_P177826	*IL6*	Interleukin 6 (interferon, beta 2)	1·03	0·37*	0·25	− 0·41*
A_72_P680394			1·12	0·30*	0·05	− 0·44*
A_72_P232367	*IL8*	Interleukin 8	5·46	5·28*	2·07	2·28†
A_72_P413808			4·84	4·57†	1·78	1·83
A_72_P177721	*IRF1*	Interferon regulatory factor 1	1·32	1·05*	0·24	0·48†
A_72_P536281			1·26	1·08†	0·39	0·59
A_72_P288509	*LTB*	Lymphotoxin beta (TNF superfamily, member 3)	1·43	1·37	1·20	0·70*
A_72_P077951	*MADCAM1*	Mucosal vascular addressin cell adhesion molecule 1	0·49	0·69†	1·01	0·54*
A_72_P444240	*MX2*	Myxovirus (influenza virus) resistance 2 (mouse)	0·29	0·57	1·07	1·05
A_72_P600868	*NFKBIA*	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1·02	0·74*	0·84	0·43*
A_72_P342398			1·16	0·86*	1·02	0·62*
A_72_P177731	*OASL*	2′-5′-Oligoadenylate synthetase-like	0·06	− 0·06	1·18	1·15
A_72_P247587	*ORM1*	Orosomucoid 1	− 0·67	− 0·50	1·23	0·71*
A_72_P088191	*PGLYRP2*	Peptidoglycan recognition protein 2	4·85	4·24*	3·72	2·62*
A_72_P367993			1·12	0·43*	0·43	0·02*
A_72_P428729	*S100A8*	S100 calcium-binding protein A8	2·04	2·07	1·74	0·98*
A_72_P137861	*S100A9*	S100 calcium-binding protein A9	1·35	1·45†	1·50	0·70*
A_72_P165616	*SAA2*	Serum amyloid A2	5·44	4·73*	5·18	4·58*
A_72_P766152			4·51	3·98*	4·32	3·63*
A_72_P760975			4·09	3·56*	3·93	3·17*
A_72_P759880			4·99	4·35*	4·83	4·18*
A_72_P441669			5·38	4·77*	4·99	4·53*
A_72_P367438	*SOCS3*	Suppressor of cytokine signalling 3	1·87	2·00	0·94	0·53†
A_72_P185956			1·03	0·97	0·38	0·11
A_72_P206202	*SPP1*	Secreted phosphoprotein 1	1·24	1·50	0·70	1·01
A_72_P442276	*STAT5A*	Signal transducer and activator of transcription 5A	1·01	0·54*	− 0·30	0·09†
A_72_P088576	*VCAM1*	Vascular cell adhesion molecule 1	2·96	2·74*	0·27	0·00

* Colostrum whey treatment significantly affects HK bacteria induction ($P<0·05$).
† Colostrum whey treatment affects HK bacteria induction ($P<0·1$).
‡ Fold change in gene expression compared with control cells is expressed on the $\log_2$ scale.

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Table 3. List of genes uninvolved in immune and/or inflammatory responses exhibiting increased expression in porcine intestinal epithelial cells after 2 and 24 h of treatment with heat-killed bacteria (HK b), without or with colostrum whey (col), as determined by the microarray analysis‡

			Fold change after 2 h of treatment		Fold change after 24 h of treatment	
Probe name	Gene symbol	Gene name	HK b	HK b + col	HK b	HK b + col
A_72_P424589	*AFP*	Alpha-fetoprotein	−1·07	−0·22*	1·16	0·08*
A_72_P035338	*AMCF-II*	Alveolar macrophage-derived chemotactic factor-II	2·83	2·23*	2·29	1·73*
A_72_P097481	*ANKRD37*	Ankyrin repeat domain-containing protein 37-like	−0·19	−0·51*	1·08	0·92
A_72_P474198	*CEBPD*	CCAAT/enhancer-binding protein delta-like	1·00	0·83*	0·23	0·21
A_72_P418444	*CFLIP*	Cellular FLICE-like inhibitory protein	1·04	1·39*	−0·02	0·26†
A_72_P223362	*CYP1A1*	Cytochrome P450 1A1	2·02	1·99	−2·82	−1·95*
A_72_P443804			1·90	1·83	−2·73	−1·51*
A_72_P755349	*CYP3A29*	Cytochrome P450 3A29	1·17	1·10	1·52	1·39
A_72_P742947	*CYP3A46*	Cytochrome P450 3A46	1·30	1·35	1·71	1·17*
A_72_P743157			1·27	1·14	1·47	1·28
A_72_P322704			1·40	1·36	1·34	1·28
A_72_P737020			1·15	1·17	1·72	1·53
A_72_P580852	*CYR61*	Cysteine-rich, angiogenic inducer, 61	1·06	1·39*	−0·11	0·34*
A_72_P319303			1·13	1·41†	−0·14	0·17†
A_72_P688281			1·09	1·49*	−0·03	0·29
A_72_P489452	*DDIT4*	DNA damage-inducible transcript 4 protein-like	0·04	0·69*	1·97	2·92*
A_72_P538933			−0·03	0·67*	1·91	2·80*
A_72_P739530	*FETUIN*	FETUIN protein	−0·88	−0·46†	1·03	1·23
A_72_P146986	*GADD45A*	Growth arrest and DNA damage-inducible, alpha	1·01	1·98*	0·24	0·57*
A_72_P409208	*GDF15*	Growth differentiation factor 15	0·96	1·22†	1·18	1·27
A_72_P303284	*HAS3*	Hyaluronan synthase 3	1·05	1·89*	−0·94	−0·32*
A_72_P223937	*ICAM-1*	Intercellular adhesion molecule-1	1·06	0·76†	0·15	0·00
A_72_P031471	*ISG12(A)*	Putative ISG12(a) protein	0·19	0·00†	1·20	0·99*
A_72_P351213	*MFSD2A*	Major facilitator superfamily domain-containing 2A	1·01	1·14	0·22	0·15
A_72_P592239	*MYC*	v-Myc myelocytomatosis viral oncogene homolog	1·26	1·08*	0·19	0·62*
A_72_P165831			1·30	1·26	0·36	0·70*
A_72_P557580			1·22	1·35	0·48	0·73
A_72_P088816	*PAG6*	Pregnancy-associated glycoprotein 6	0·13	−0·21*	1·15	0·47*
A_72_P709245			0·29	−0·02	1·22	0·71†
A_72_P692939	*PIAP*	Inhibitor of apoptosis-like	1·75	1·88	0·49	0·35
A_72_P088296			1·78	1·74	0·18	0·23
A_72_P010246	*PLAU*	Plasminogen activator, urokinase	1·36	1·81*	0·28	0·86†
A_72_P770588	*PLET*	Placenta expressed transcript protein	1·63	1·38	0·49	0·88*
A_72_P088242			1·69	1·28*	0·68	0·80
A_72_P728098			1·48	0·95*	0·44	0·65
A_72_P261022	*PPP1R3C*	Protein phosphatase 1, regulatory subunit 3C	−0·12	−0·02†	1·11	1·28
A_72_P165926	*RGS4*	Regulator of G-protein signalling 4	1·40	1·82*	1·30	1·88*
A_72_P337738	*RHOV*	Ras homolog gene family, member V	1·18	1·00	0·23	0·44
A_72_P276754	*SLC2A6*	Solute carrier family 2, member 6	1·72	1·51*	0·24	−0·56*
A_72_P036711	*TIPARP*	TCDD-inducible poly(ADP-ribose) polymerase	1·28	1·66*	−0·87	−0·63
A_72_P024051			1·15	1·72*	−0·81	−0·81

* Colostrum whey treatment significantly affects HK bacteria induction ($P<0·05$).
† Colostrum whey treatment affects HK bacteria induction ($P<0·1$).
‡ Fold change in gene expression compared with control cells is expressed on the $\log_2$ scale.

as evidenced by a decrease in the expression of most of the inflammatory genes induced by HK bacteria. Similar observations were also made in human colon carcinoma Caco-2/15 cells, in which a decrease in the expression and secretion of *IL8*, *CCL2* and *CXCL10* after colostrum whey treatment was measured using, respectively, the q-PCR analysis and ELISA (data not shown).

Colostrum whey decreases the activation of NF-κB induced by heat-killed bacteria

NF-κB is a transcription factor that plays a major role in the cellular response to a wide variety of stimuli, including pathogens such as ETEC and *Salmonella*[37]. The effect of colostrum whey treatment on the activation of NF-κB induced by HK bacteria was investigated. The results obtained in both IPEC-J2 and Caco-2/15 cells revealed that colostrum whey treatment decreased the HK bacteria-dependent induction of NF-κB-luc activity after 6 h (Fig. 4). Interestingly, treatment with other milk fractions, including cheese whey as well as two milk components, namely lactoferrin and caseinomacropeptide, failed to decrease NF-κB-luc activity induced by HK bacteria in IPEC-J2 and Caco-2/15 cells. A dose-dependent decrease in NF-κB-luc activity induced by HK bacteria was observed in the colostrum whey-treated Caco-2/15 cells (Fig. 5(a)). This dose-dependent decrease in the HK bacteria-dependent induction caused by colostrum whey treatment was also observed using the inflammatory reporter constructs IL-8-luc and IL-6-luc (Fig. 5(b) and (c)).

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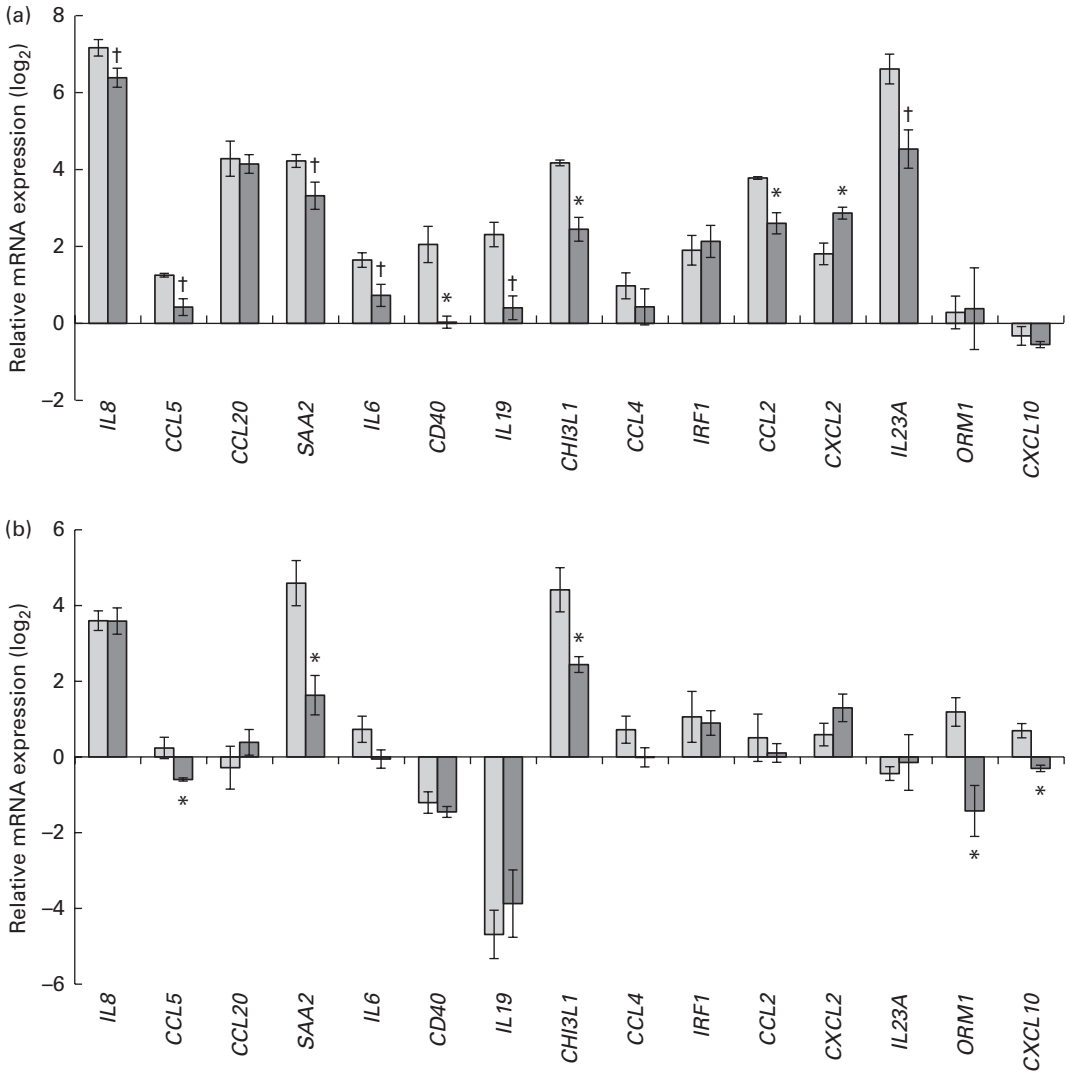

Fig. 3. Results of the quantitative PCR analysis of the expression of immune and inflammatory genes induced by heat-killed (HK) bacteria in porcine intestinal epithelial cells (IPEC-J2) incubated with or without colostrum whey. IPEC-J2 were treated with HK bacteria with colostrum (■) or without colostrum (□) whey for (a) 2 h and (b) 24 h, followed by RNA extraction. The following genes were selected from the microarray analysis: *IL8*; chemokine (C-C motif) ligand 5 (*CCL5*); *CCL20*; serum amyloid A2 (*SAA2*); *IL6*; CD40 molecule, TNF receptor superfamily member 5 (*CD40*); *IL19*; chitinase 3-like 1 (*CHI3L1*); *CCL4*; interferon regulatory factor 1 (*IRF1*); *CCL2*; chemokine (C-X-C motif) ligand 2 (*CXCL2*); *IL23A*; orosomucoid 1 (*ORM1*); *CXCL10*. mRNA expression level on the $\log_2$ scale was calculated by comparing the expression levels of treated cells with untreated (control) cells, using the $\Delta\Delta C_T$ method and β-actin as the internal control. Values are means for three independent experiments, with their standard errors represented by vertical bars. * Mean value was significantly different from that of the untreated cells ($P<0{\cdot}05$). † Mean value was different from that of the untreated cells ($P<0{\cdot}1$).

Discussion

The beneficial effects of colostrum on intestinal epithelial function and development are well known and the protection it gives to newborns against enteric infections has been observed in a number of studies[22,38–40]. In addition, the importance of colostrum for the development of the immune system is well established[41,42]. However, the molecular mechanisms involved in the colostrum regulation of intestinal inflammation caused by pathogenic invasion remain poorly understood. The results of the present study showed that bovine colostrum whey treatment specifically decreased the expression of inflammatory genes and the activation of the NF-κB signalling pathway in intestinal epithelial cells exposed to pathogenic bacteria.

Although the release of pro-inflammatory chemokines and cytokines by intestinal epithelial cells during infections is crucial because it triggers the recruitment of neutrophils, macrophages and dendritic cells to the site of infection, which in turn initiate innate and adaptive immune responses, it leads to an increase in neutrophil extravasation and vascular permeability, eventually leading to tissue damage and fluid loss in the intestinal lumen[2–4]. In fact, the induction of inflammatory gene expression in intestinal epithelial cells after stimulation with ETEC or *Salmonella*, as well as a decrease in transepithelial electrical resistance associated with increased paracellular permeability, has been described in many studies[5,6,12,13,43–49]. Indeed, the results of the microarray analysis carried out in the present study showed that the expression of many genes

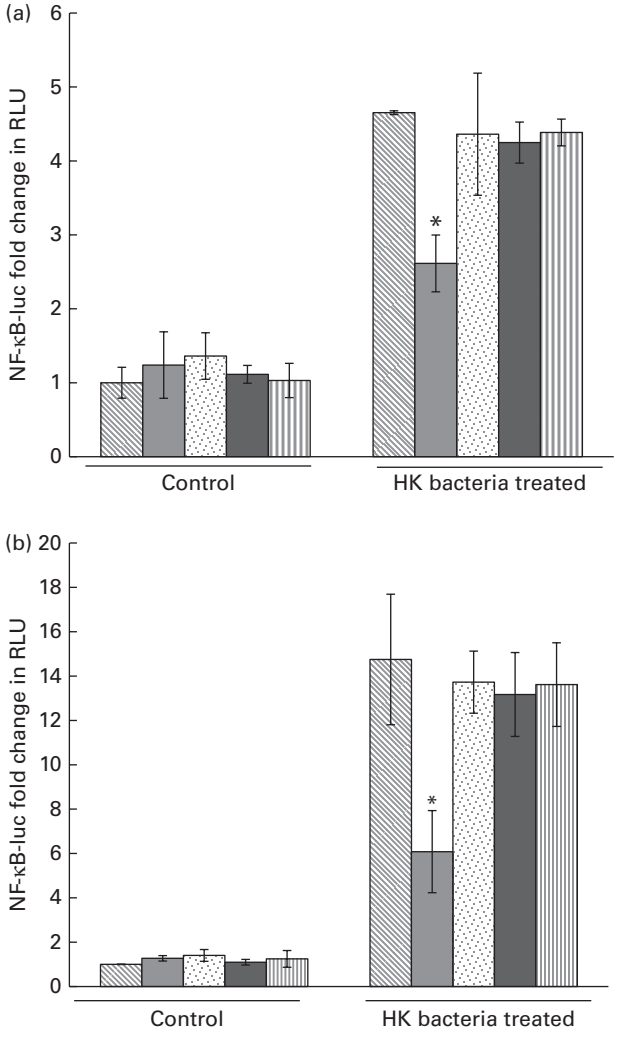

Fig. 4. Effect of colostrum whey on NF-κB-luc activity induced by heat-killed (HK) bacteria in intestinal epithelial cells. (a) Porcine intestinal epithelial cells and (b) human Caco-2/15 cells were transiently transfected with the NF-κB-luc reporter construct. Cells were treated with HK bacteria with or without milk fractions. Values are means for three independent experiments, with their standard errors represented by vertical bars. Significant decreases in NF-κB-luc activity induced by milk fractions were determined by comparing the relative luciferase activity in cells treated with HK bacteria and milk fractions with that in cells treated with HK bacteria. * Mean value was significantly different from that of the untreated cells ($P<0{\cdot}05$). ▨, Untreated; ■, 10 mg/ml colostrum whey; ⚀, 10 mg/ml cheese whey; ■, 1 mg/ml lactoferrin; ▥, 1 mg/ml caseinomacropeptide. RLU, relative light units.

involved in the regulation of cell death, including *CCL5*, *CD40*, *IL19*, *IL6*, *IRF1*, signal transducer and activator of transcription 5A (*STAT5A*), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α (*NFKBIA*), and S100 calcium-binding proteins A8 and A9 (*S100A8* and *S100A9*), in IPEC-J2 was rapidly induced after stimulation with HK bacteria and colostrum whey treatment decreased the induction of their expression. Together, these results suggest that colostrum whey might act by limiting the inflammatory processes in cells and preventing destructive processes induced by pathogens. Indeed, studies carried out in premature infants have shown that lactoferrin, as well as other specific nutrients, preserve the integrity of mucosal barrier by modulating host defence mechanisms and bacterial translocation[50].

Previous studies have shown that milk components inhibit bacterial activity by preventing bacterial growth and

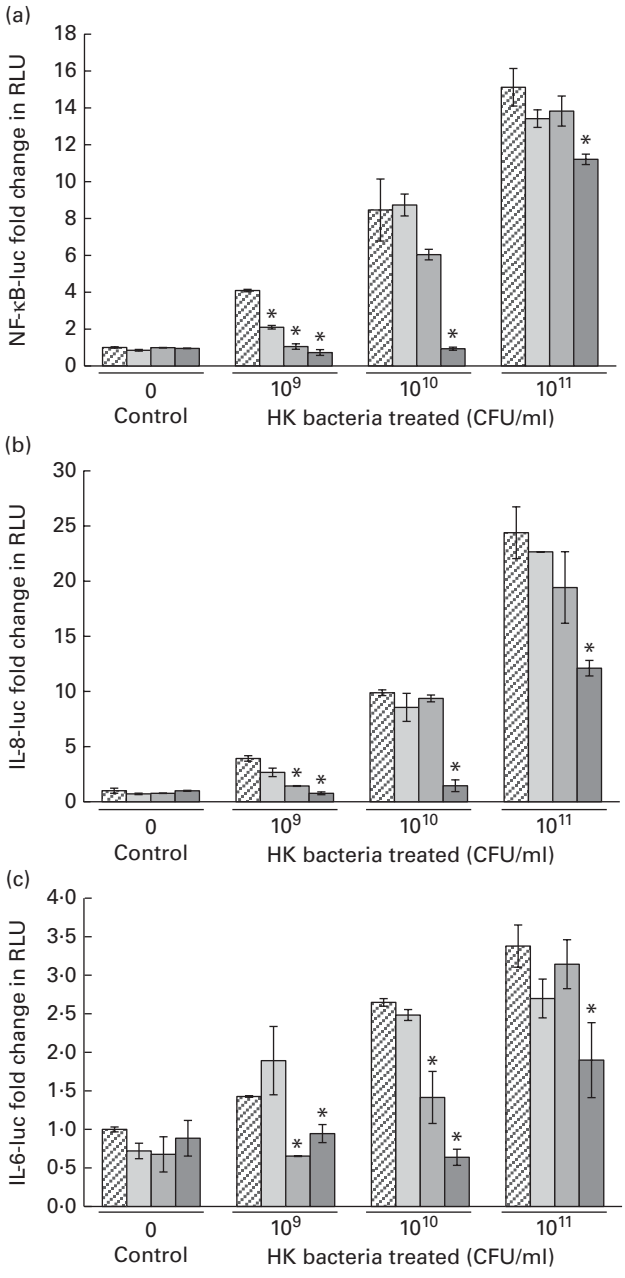

Fig. 5. Effect of colostrum whey on NF-κB-luc, IL-8-luc and IL-6-luc reporter activity in Caco-2/15 human colon carcinoma cells induced by heat-killed (HK) bacteria. Caco-2/15 cells were transiently transfected with the (a) NF-κB-luc, (b) IL-8-luc or (c) IL-6-luc reporter constructs. Cells were treated with different amounts of HK bacteria (0 colony-forming units (CFU)/ml (control), 10^9 CFU/ml, 10^{10} CFU/ml and 10^{11} CFU/ml) without (⚀, untreated) or with increasing concentrations of colostrum whey (□, 0·1 mg/ml; ■, 1 mg/ml; ■, 10 mg/ml). Values are means for three independent experiments, with their standard errors represented by vertical bars. Significant decreases in NF-κB-luc, IL-8-luc and IL-6-luc activity mediated by colostrum whey were determined by comparing the relative luciferase activity in cells treated with both HK bacteria and colostrum whey with that in the respective HK bacteria-treated cells. * Mean value was significantly different from that of the untreated cells ($P<0{\cdot}05$). RLU, relative light units.

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cell binding[30,51–54]. Therefore, colostrum whey may indirectly inhibit the inflammatory responses of intestinal epithelial cells by blocking the interaction between bacterial components and epithelial cell membrane receptors. In fact, the anti-inflammatory effect exerted by colostrum whey required the simultaneous presence of bacterial components, as preincubation of cells with colostrum whey failed to block the activation of NF-κB induced by further stimulation with bacteria, unless colostrum whey remained in the medium during bacterial stimulation (data not shown). In addition, the activation of NF-κB-luc was not decreased when colostrum whey was added 4 h after stimulation with HK bacteria.

Recent results obtained in our laboratory have shown that colostrum whey also exerts beneficial effects directly on intestinal epithelial cells by promoting proliferation and wound-healing processes[55]. Indeed, the exposure of intestinal epithelial cells to colostrum whey alone rapidly induces the expression of a number of genes, including colony-stimulating factor 2 (*CSF2*), cytochrome P450 1A1 (*CYP1A1*), cysteine-rich angiogenic inducer, 61 (*CYR61*), growth arrest and DNA damage-inducible, α (*GADD45A*), hyaluronan synthase 3 (*HAS3*), *IL8*, plasminogen activator, urokinase (*PLAU*) and TCDD-inducible poly(ADP-ribose) polymerase (*TIPARP*), which are also implicated in the cellular response to bacterial components as observed in the present study. Other studies have shown that bovine colostrum exerts protective effects against necrotising enterocolitis and formula-induced inflammation in preterm pigs[29,39] and promotes tissue recovery following intestinal damage in mice[40]. In addition, a study performed in weaned pigs has shown that intestinal weight and villus height are affected by bovine colostrum[56]. Thus, the protective role of colostrum whey in the intestinal epithelium is likely to go beyond preventing the attachment of pathogens to the epithelium, but also promotes directly health and integrity of intestinal epithelium by regulating cellular processes.

Interestingly, cheese whey and other components obtained from bovine milk, including lactoferrin and caseinomacropeptide, failed to decrease the induction of NF-κB-luc activity. This was also observed in our recent study, in which colostrum was found to promote the migration of intestinal epithelial cells, while cheese whey at a similar concentration failed to do so[55]. Therefore, a unique component of colostrum whey or the precise composition and proportion of colostrum whey components seem to be critical for the responses of intestinal epithelial cells to pathogens. Indeed, immune and antibacterial components found specifically in colostrum whey might be required to attenuate immune and inflammatory activation through the NF-κB signalling pathway. In addition, the proportion of these beneficial molecules is important as a dose-dependent decrease in HK bacteria-induced NF-κB-luc activity was observed in the colostrum whey-treated Caco-2/15 cells.

Conclusion

Colostrum contains a wide variety of immunoregulatory molecules that could counteract numerous intestinal diseases, including bacterial or viral gastroenteritis and inflammatory bowel disease. The results of the present study indicate that colostrum treatment decreases inflammatory responses and inflammatory gene expression induced by ETEC and *Salmonella* components in the intestinal epithelial cells. This colostrum whey-dependent reduction of inflammatory gene expression is associated with a reduction of NF-κB activation. The results also indicate that colostrum can control inflammatory processes in intestinal epithelial cells in the presence of pathogens by decreasing the ability of bacteria to bind to epithelial cells and/or by acting directly on intestinal epithelial cells, promoting the growth and integrity of the epithelium while moderating inflammatory signalling pathways. Therefore, colostrum whey holds great potential as a feed additive for preventing gastrointestinal infections.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114514003481

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The authors' contributions are as follows: M. L., Y. P., S. F. G. and Y. B. conceived and designed the study; Y. P. and S. F. G. prepared the colostrum whey as well as cheese whey samples; C. A. provided the luciferase constructs; M. B. conducted all the experiments; M. F. performed the luciferase assay in Caco-2/15 cells; M. B. analysed and interpreted the data, under the direction of M. L.; M. B. wrote the manuscript; M. L., C. A., Y. P., S. F. G. and Y. B. revised the manuscript.

None of the authors has any competing interests to declare.

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