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Peptides encrypted in the human intestinal microbial-exoproteome as novel biomarkers and immunomodulatory compounds in the gastrointestinal tract

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ABSTRACT

Peptides encrypted in the intestinal microbial-exoproteome mediate the host-microbiota crosstalk, which is disrupted in inflammatory bowel disease (IBD). Here, the MAHMI database was used for the identification of 20 novel intestinal bacterial peptides. Our results revealed that serum IgA levels directed towards the peptides, but not IgG, discriminated healthy controls from IBD patients. Indeed, they also differentiated patients with ulcerative colitis from Crohńs disease and, within them, patients with and without intestinal inflammation. All peptides were immunomodulatory as they changed the intestinal cytokine milieu following human lamina propria mononuclear cells culture (with/out LPS), revealing a *Bifidobacterium longum* subsp. *longum* peptide with the highest tolerogenic properties. Therefore, bacterial peptides encrypted in the human gut metaproteome may have utility as non-invasive biomarkers to aid on IBD diagnosis and monitoring. These peptides also display immunomodulatory effects on the intestinal mucosa revealing them as novel functional compounds for non-drug therapeutic strategies in IBD.

1. Introduction

Inflammatory bowel disease (IBD) comprises a group of chronic inflammatory disorders of the gastrointestinal tract mainly classified into Crohńs disease (CD) and ulcerative colitis (UC). Despite its precise etiology remains unknown, a complex combination of genetic, environmental, immunologic and microbial factors might be involved in this disease. Host-microbe interactions, microbiota recognition defects and intestinal dysbiosis are key factors in the immunopathogenesis of IBD (Wallace, Zheng, Kanazawa, & Shih, 2014). There is growing evidence about the immune modulation capability of bioactive components within the intestinal lumen. Indeed, strategies about dietary and microbiota-derived compounds are becoming a potential alternative for non-drug therapeutic intervention in IBD (Derwa, Gracie, Hamlin, & Ford, 2017; Larussa, Imeneo, & Luzza, 2017). Unravelling this intricate network at the intestinal mucosa milieu offers therefore a novel challenge for the diagnosis and treatment of these disorders (Rapozo, Bernardazzi, & de Souza, 2017).

Anti-inflammatory, antioxidant and immunomodulatory compounds as well as probiotics and prebiotics agents have demonstrated benefits at maintaining gut homeostasis in the context of IBD (Dolan & Chang, 2017; Montalban-Arques et al., 2015; Statovci, Aguilera,

Abbreviations: CD, Crohńs disease; HC, healthy controls; IBD, inflammatory bowel disease; LPMC, lamina propria mononuclear cells; MAHMI, Mechanism of Action of the Human Microbiome; PBMC, peripheral blood mononuclear cells; UC, ulcerative colitis

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Table 1
Bacterial peptides used in the study and information associated.

Peptide	Sequence	Uniprot organism	Bacteria type	Uniprot ID	Uniprot protein	Uniprot gene	Bioactivity (%)
B1	YSLPKSIANSNNIGGIA	*Ruminococcus albus*	Commensal	P16216	GUN1_RUMAL Endoglucanase 1	Eg I	100
B2	NGHRTERLPNNVN	*Ruminococcus flavefaciens*	Commensal	O54055	ISCS_RUMFL Cysteine desulfurase IscS	iscS	75
B3	DEIDIEILGKDTTKVQF	*Ruminococcus flavefaciens*	Commensal	Q53317	XYND_RUMFL Xylanase/beta-glucanase	xynD	75
B4	GTSFEGNEYFEGPSIR	*Clostridium stercorarium*	Transient/Food	P48790	XYLA_CLOSR Xylosidase/arabinosidase	xylA	100
B5	AIQDEIDQLSTEIDR	*Clostridium tyrobutyricum*	Transient/Food	P80583	FLA_CLOTY Flagellin (Fragment)	fla	75
B6	FAIVDEVDSILIDEAR	*Bifidobacterium longum*	Commensal/Probiotic	B3DR89	SECA_BIFLD Protein translocase subunit SecA	secA	100
B7	WIEAVGYSLTQHPDPELEK	*Bifidobacterium longum* subsp. *longum*	Commensal/Probiotic	E8MGH8	HYBA1_BIFL2 Non-reducing end beta-L-arabinofuranosidase	hypBA1	100
B8	TGEGILALAGDNTYK	*Serratia marcescens*	Pathogen	P09489	PRTS_SERMA Extracellular serine protease	Unknown	75
B9	ENIRDANNNRTPEERREL	*Staphylococcus epidermidis*	Opportunistic pathogen	Q8CP76	EBH_STAES Extracellular matrix-binding protein ebh	ebh	75
B10	NAALASYSLASDLDR	*Escherichia coli O78:H11*	Pathogen	Q9XD84	TIBA_ECOH1 Adhesin/invasin TibA autotransporter	tibA	100
B11	MTPANEARPIELAFK	*Bifidobacterium bifidum*	Commensal/Probiotic	D4QFE7	BGAL_BIFBI Beta-galactosidase BbgII	bbgII	75
B12	LPLAFFVLTFLWALILR	*Bacteroides fragilis*	Opportunistic pathogen	Q3V815	MTGA_BACFR Monofunctional biosynthetic peptidoglycan transglycosylase	mtgA	80
B13	ASPEDEAASMEKAKAF	*Bacteroides vulgatus*	Commensal	A6L1Z2	G1095_BACV8 Glycosyl hydrolase family 109 protein 5	BVU_2041	80
B14	WPTTEIYPDSPWNYSLVLDK	*Bifidobacterium longum* subsp. *longum*	Commensal/Probiotic	E8MGH8	HYBA1_BIFL2 Non-reducing end beta-L-arabinofuranosidase	hypBA1	100
B15	VDLVDWNSHDNAEVWR	*Bifidobacterium longum*	Commensal/Probiotic	Q8GN32	MOBA_BIFLO Probable mobilization protein MobA	mobA	75
B16	ATQERIPKDSF	*Bifidobacterium breve*	Commensal/Probiotic	P94248	BGLFU_BIFBR Bifunctional beta-D-glucosidase/beta-D-fucosidase	Unknown	75
B17	HIGVHSDPVDK	*Butyrivibrio fibrisolvens*	Commensal	P30269	AMY_BUTFI Alpha-amylase	amyA	75
B18	SLANYSTVPSFASAIASK	*Lactobacillus delbrueckii* subsp. *bulgaricus*	Commensal/Probiotic	Q04BA5	ATPA_LACDB ATP synthase subunit alpha	atpA	75
B19	VPSEKKAELF	*Lactobacillus helveticus*	Probiotic	P38059	SLAP_LACHE S-layer protein	slpH	80
B20	SDGYSYSLYGYMNVETK	*Lactococcus lactis* subsp. *cremoris*	Transient/Food	A2RKH5	SCPA_LACLM Segregation and condensation protein A	scpA	100

MacSharry, & Melgar, 2017; Uranga, Lopez-Miranda, Lombo, & Abalo, 2016). Likewise, microbiota might regulate intestinal immunity not only by direct contact with mucosal systems but also by soluble chemical mediators (Belkaid & Naik, 2013). Recently, the concept of probiotic-produced soluble factors has been presented as an interesting niche towards the development of functional foods, especially in chronic conditions such as IBD (Sánchez, Urdaci, & Margolles, 2010). It has been suggested that probiotic functionality might be driven through bacterial secreted metabolites and/or isolated surface compounds rather than by changes in microbial populations (Sanchez et al., 2017). For instance, intestinal SCFA produced by gut microbiota from dietary fiber induced the differentiation of colonic Treg cells via FoxP3 (Furusawa et al., 2013). This homeostatic role is nevertheless impaired in IBD where active patients display reduced levels of fecal SCFA (Treem, Ahsan, Shoup, & Hyams, 1994) coupled with lower levels of SCFA-producing bacteria (Treem et al., 1994).

Extracellular bacterial proteins and encrypted peptides released by proteolysis have been also proposed as key agents in the host/microbiota interplay given their immunomodulatory capacity and effects on the maintenance of mucosal barrier (Ruiz et al., 2016; Sanchez, Bressollier, & Urdaci, 2008). Indeed, beyond their bioactivity, these extracellular bacterial peptides might serve as biomarkers of gut homeostasis as well. Hence, the serine-threonine peptide STp secreted by *Lactobacillus plantarum* is mostly present in the colon of healthy controls (HC) but not in active UC patients, showing *ex vivo* regulatory effects on intestinal dendritic cells via IL-10 towards an anti-inflammatory profile in the UC mucosa (Al-Hassi et al., 2014; Bernardo et al., 2012). In order to get a deeper insight into the *in silico* screening of other immunomodulatory and anti-proliferative peptides produced by the human gastrointestinal microbiota, the "Mechanism of Action of the Human Microbiome" (MAHMI) database (http://www.mahmi.org) has been recently developed (Blanco-Miguez, Gutierrez-Jacome, Fdez-Riverola, Lourenco, & Sanchez, 2017). Through MAHMI prediction, Hidalgo-Cantabrana and colleagues (Hidalgo-Cantabrana et al., 2017) demonstrated the capacity of 15 novel peptides to *ex vivo* modulate immune responses on human peripheral blood mononuclear cells (PBMC). Indeed, they concluded that bacteria-secreted peptides FR-16 from *Bifidobacterium longum* DJ010A and LR-17 from *Bifidobacterium fragilis* YCH46 displayed the higher immunomodulatory potential (Hidalgo-Cantabrana et al., 2017). Nevertheless, the effect of those peptides on the human gastrointestinal tract remains unknown.

In this study, we therefore aimed to investigate the biomarker potential and immunomodulatory capacity of FR-16 and LR-17 peptides in the human gastrointestinal tract. Moreover, we also studied the effect of 7 other peptides previously studied on PBMC together with 11 novel peptides which had not been previously described, aiming to identify novel immunomodulatory compounds in the human gastrointestinal tract.

2. Material and methods

2.1. Peptide identification and bioactivity prediction

Bacterial peptides were identified through the MAHMI database (http://www.mahmi.org) (Blanco-Miguez et al., 2017). MAHMI integrates a curated protein database including immunomodulatory proteins, as well as the content of the VaxinPAD server of peptide-based vaccine adjuvants (Nagpal et al., 2015) and the CancerPPD database on anti-proliferative peptides and proteins (Tyagi et al., 2015). All peptides were generated by enzyme digestion with the major human intestinal endoproteases and processed to predict any possible bioactivity using ClustalΩ. Any peptide is considered as potentially bioactive if its amino acid similarity with experimentally validated peptides is higher than 60% according to ClustalΩ. Nevertheless, and in order to perform a more rigorous selection, in our approach we decided to consider a similarity threshold of 75%. Therefore, 20 novel peptides encrypted

within larger proteins secreted from microbial organisms present in the human gastrointestinal tract and which are released by digestive enzymes were identified and chemically synthetized. Table 1 shows the sequence of the selected peptides, as well as the protein and organism from which they are derived and their potential percentage of bioactivity. Additional information about physiochemical properties of peptides is shown in the Supporting Information Table S1. All experiments were performed in comparison with the immunomodulatory peptide STp (Al-Hassi et al., 2014; Bernardo et al., 2012). LPS-free chemically synthesized peptides (> 95% purity in all cases) were purchased from Genecust facilities (Ellange, Luxemburg). Freeze-dried peptides were stored at −80 °C until used.

2.2. Patients and biological samples

Serum samples were obtained from the biological collection available in our research facilities (registered on the Spanish Registry of Biological Collections and Biobanks -C.0003482) including samples from HC with no known autoimmune disease or malignancy, and IBD patients (Supporting Information Table S2). IBD serum samples included both UC and CD, and were obtained at the time that colonoscopy was performed due to clinical practise for disease diagnosis or monitoring. That allowed us to categorize IBD samples into active (inflamed) or quiescent (non-inflamed) as defined by the endoscopic assessment. Hence, UC patients were divided into active (Mayo endoscopic score > 1) and quiescent (Mayo endoscopic score ≤1), while CD patients were divided into active (simplified endoscopic activity (SES-CD) score > 3) and quiescent CD (SES-CD score ≤3).

Colonic biopsies were obtained during colonoscopy from a total of 8 HC (Supporting Information Table S3). Patients had been referred due to rectal bleeding, dyspepsia or colorectal cancer screening. In all cases, they had macroscopically and histologically normal mucosa. Samples were immediately preserved in ice-chilled complete medium [Dutch modified RPMI 1640 (Sigma-Aldrich, Dorset, UK) containing 100 μg/mL penicillin/streptomycin, 2 mM L-glutamine, 50 μg/mL gentamicine (Sigma-Aldrich) and 10% foetal calf serum (TCS cellworks, Buckingham, UK)] and processed within 30 min. In all cases, samples were obtained following informed consent after ethical approval from the Ethics Committee at La Princesa Hospital.

2.3. Serum IgG and IgA measurement towards bacterial peptides

IgG and IgA levels were measured by home-made ELISA directed against the 20 peptides. Briefly, each peptide was used in a dilution of 1:1000 in PBS on a final volume of 200 μL per well on a 96 well-plate which was incubated at 4 °C overnight. Next day, the plates were washed 5 times with 300 μL PBS-Tween 0.05% (v/v). The plate was further blocked with 200 μL BSA 1% (v/v) in PBS and incubated for 1 h in gentle shaking at room temperature, followed by a further washing step as previously described. Following serum incubation, wells were further washed before addition of the secondary antibodies [anti-human IgG or anti-human IgA conjugated with horseradish peroxidase, 5 μg/ml each (Sigma Aldrich)]. Wells were washed again before addition of 100 μL tetramethylbenzidine for 7 min, prior to stop the reaction with 50 μL H_2SO_4 (2 M). OD were measured at λ = 450 nm. For each of the 20 peptides, a total of 10 pooled sera per group (HC, active CD, active UC, quiescent CD and quiescent UC) were evaluated in duplicate, for a total of 7 serial dilutions of serum (1/50; 1/100; 1/200; 1/400; 1/800, 1/1600 and 1/3200) for a period of 2 h (gentle shaking, room temperature). Dilution 1/50 was selected as the optimal one, being therefore subsequently used to identify the differences on the antibody levels (IgG and IgA) directed towards peptides allowing us to compare (i) patients with IBD and HC; (ii) patients with CD and UC; and within CD and UC, (iii) patients with endoscopically active and quiescent disease.

2.4. Biopsy culture

Colonic biopsies were processed as previously described (Bernardo et al., 2016). Briefly, biopsies were processed to obtain lamina propria mononuclear cells (LPMC) following two incubations (30 min each) with Hanks balanced salt solution (Gibco BRL, Paisley, Scotland, UK) containing 1 mM DTT and 1 mM EDTA solutions to remove the associated mucus/bacteria and epithelial layer, respectively. Biopsy digestion was performed in the presence of 1 mg/mL of collagenase D and 20 μg/mL of liberase (Roche Diagnostics Ltd, Lewes, UK). LPMC were subsequently passed through a 100 μm cell strainer and collected by centrifugation before they were cultured overnight (200.000 cells in 200 μL) in medium supplemented with/out each of the peptides (1 μg/mL) in the presence/absence of LPS (100 ng/ml, Sigma-Aldrich). All 44 culture conditions (basal, STp and each of the 20 peptides in the presence/absence of LPS) were performed using LPMC from the same HC hence allowing us to perform paired cultures.

2.5. Determination of the lamina propria cytokine milieu

Cell-free culture supernatant from the LPMC were collected and immediately criopreserved at −80 °C. Prior to analysis, samples were thawed and centrifuged to remove any debris hence ensuring that all culture supernatants were processed in the same manner and had undergone just once cycle of thawing before cytokine determination. Seven cytokines (IFN-γ, IL-10, IL-17A, IL-1β, IL-6, TNF-α, and IL-8) were measured using MILLIPLEX® MAP custom magnetic bead panel kit (Supporting Information Table S4) following the manufacture's specifications. A broad sensitivity range of standards were used to help enable the quantitation of a wide dynamic range of cytokine concentrations while still providing high sensitivity (Supporting Information Table S5). Median fluorescent intensity was calculated using the MAGPIX® system version (Luminex) which integrates the Luminex xPOTENT® acquisition software and the MILLIPLEX® Analyst 5.1 analysis software.

2.6. Statistical analysis

Data were analysed using GraphPad Prism 6.01 software (San Diego, CA, USA) by One-way ANOVA, followed by Tukey or Fisheŕs LSD ad-hock test as detailed in the figure legends. P value < 0.05 were considered statistically significant.

3. Results & discussion

Host-microbiota interactions are essential to maintain the mechanisms of intestinal immune homeostasis. Nevertheless, this dialogue is altered in IBD patients, where there is both a reduced bacterial diversity and an altered mucosal composition. In this context, it has been recently proposed that the host-microbiota crosstalk is mediated by bioactive bacteria-secreted peptides, which may also have utility as biomarkers (Ruiz, Hevia, Bernardo, Margolles, & Sanchez, 2014; Tsilingiri et al., 2012). In order to get a deeper insight into that mechanism, here we have used the MAHMI pipeline (Hidalgo-Cantabrana et al., 2017) for the screening of novel peptides encrypted in extracellular proteins of the human gut microbiome. All selected peptides were extracellular (either secreted or present on the bacteria surface), showed *in silico* resistance against gastrointestinal digestion and displayed bioactivity prediction equal or higher to 75% (Table 1). Of them, 9 peptides had been previously described and assayed for bioactivity on a model of PBMC, with two of them revealing a clear immunomodulatory capacity (Hidalgo-Cantabrana et al., 2017). The remaining 11 peptides had never been previously described to our knowledge. Moreover, we also screened whether these peptides may be used as non-invasive IBD biomarkers by measuring human serum IgG and IgA levels directed towards them, at the time that we determined

their potential immunomodulatory role at the intestinal mucosa, both in resting conditions as well as in the presence of a pro-inflammatory insult.

3.1. Antibody levels towards the bacterial peptides as novel IBD biomarkers

The study of circulating antibodies directed towards microbial compounds such as anti-*Saccharomyces cerevisiae* (ASCA) or *Escherichia coli* outer-membrane porin C (anti-OmpC), has gained clinical interest as novel non-invasive serum biomarkers of CD and UC (Dubinsky & Braun, 2015). However, few bacterial proteins involved in gut homeostasis have been described to date. For instance, *Faecalibacterium prausnitzii* represent the most abundant bacterium of the healthy intestinal microbiota, whose absence has been consistently associated with CD (Sokol et al., 2008). A 15 kDa protein of this bacterium, and seven derived peptides released to culture supernatants have shown anti-inflammatory properties through NF-κB signalling in intestinal epithelial cells and dinitrobenzene sulfonic acid-induced mice colitis (Quevrain et al., 2016). Interestingly, faecal microbiota analysis in UC patients has revealed higher abundance of this commensal in patients responding to the anti-TNF therapy as compared with non-responders (Magnusson et al., 2016). Therefore, the presence of bacterial antibodies in faecal and mucosal samples, as well as their correlation with endoscopic severity scores, opens a new dimension for IBD diagnosis and monitoring. In order to shed some light on those previous observations, we here assessed whether the levels of circulating antibodies directed towards these peptides may have utility as novel non-invasive IBD biomarkers.

Serum IgG antibodies levels directed against each peptide are shown on Table 2. IgG levels were higher in active UC patients compared with the other study groups with the exception of peptides B7, B10, B14, B18, B20 and STp. Serum IgG quantification was therefore useful to discriminate UC patients with active inflammation from those with non-inflammation, including HC and patients with quiescent disease, but also from active CD patients (Table 3). Referred to the CD patients, IgG levels towards the peptides only reached statistical significance in the case of peptide B20, which allowed us to categorize CD patients (active *versus* quiescent); and peptides B10, B16 and B20 which, within quiescent IBD patients, discriminated patients with CD and UC.

Regarding the IgA levels directed towards the peptides, a higher classificatory potential was found (Tables 4 and 5). This observation is in line with a previous study in which IgG titers against a cell-wall hydrolase from *Lactobacillus casei* subsp. *rhamnosus* GG were equal among HC and IBD individuals, although their IgA counterparts differentiated the groups (Hevia et al., 2014). Hence, IgA levels directed towards the peptides allowed the discrimination of HC from quiescent CD, active CD, quiescent UC and active UC (excepting B19). IgA levels allowed us to differentiate within CD patients, excluding B17, those with active disease from those who did not have intestinal inflammation. Moreover, and within non-inflamed patients, all peptides (excluding B2, B9 and B17) discriminated between patients with quiescent CD and quiescent UC. Finally, peptides B3, B14 and B15 displayed the higher biomarker potential as serum IgA levels directed against them

Table 3
Statistical differences of human serum IgG levels towards bacterial peptides.

Peptide	HC *vs* aUC	HC *vs* qUC	HC *vs* aCD	HC *vs* qCD	aUC *vs* qUC	aCD *vs* qCD	aUC *vs* aCD	qUC *vs* qCD
B1	***	ns	ns	ns	***	ns	***	ns
B2	***	ns	ns	ns	**	ns	***	ns
B3	**	ns	ns	ns	***	ns	**	ns
B4	***	ns	ns	*	****	ns	****	ns
B5	****	*	**	***	****	ns	***	ns
B6	****	ns	ns	ns	****	ns	****	ns
B7	*	ns	ns	*	ns	ns	ns	ns
B8	****	ns	ns	*	***	ns	***	ns
B9	****	*	**	***	***	ns	***	ns
B10	ns	ns	ns	ns	ns	ns	ns	*
B11	***	ns	ns	ns	****	ns	***	ns
B12	ns	**	**	*	**	ns	***	ns
B13	***	ns	ns	ns	**	ns	****	ns
B14	ns	***	***	***	**	ns	**	ns
B15	***	ns	ns	ns	***	ns	***	ns
B16	*	**	ns	ns	****	ns	**	***
B17	****	ns	ns	ns	****	ns	****	ns
B18	ns	*	*	ns	ns	ns	ns	ns
B19	ns	ns	*	*	*	ns	**	ns
B20	ns	*	ns	ns	*	***	ns	***
STp	*	**	**	***	ns	ns	ns	ns

One-way ANOVA with Tukey correction was applied for each peptide to compare groups (HC: healthy controls, aUC: active ulcerative colitis, qUC: quiescent ulcerative colitis, aCD: active Crohńs disease, qCD: quiescent Crohńs disease), and p-values < 0.05 were considered significant (* < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001).

Table 2
Human serum IgG levels towards bacterial peptides.

Peptide	HC	aUC	qUC	aCD	qCD
B1	2.488 (0.044)	2.961 (0.023)	2.408 (0.101)	2.465 (0.036)	2.492 (0.031)
B2	2.276 (0.092)	2.839 (0.037)	2.338 (0.121)	2.265 (0.036)	2.381 (0.027)
B3	2.053 (0.160)	2.426 (0.016)	1.947 (0.044)	1.959 (0.002)	2.197 (0.020)
B4	2.145 (0.025)	2.805 (0.025)	2.311 (0.096)	2.207 (0.021)	2.407 (0.022)
B5	1.828 (0.004)	2.476 (0.145)	2.051 (0.081)	2.098 (0.023)	2.150 (0.028)
B6	2.262 (0.087)	2.728 (0.067)	2.282 (0.025)	2.135 (0.033)	2.225 (0.002)
B7	2.421 (0.095)	2.823 (0.063)	2.571 (0.045)	2.694 (0.024)	2.844 (0.149)
B8	1.985 (0.049)	2.567 (0.049)	2.083 (0.039)	2.081 (0.023)	2.215 (0.053)
B9	2.001 (0.033)	2.820 (0.040)	2.277 (0.053)	2.378 (0.004)	2.442 (0.011)
B10	3.433 (0.101)	3.490 (0.021)	3.154 (0.077)	3.205 (0.084)	3.591 (0.024)
B11	2.180 (0.009)	2.598 (0.030)	2.153 (0.055)	2.214 (0.017)	2.311 (0.034)
B12	3.493 (0.074)	3.593 (0.074)	3.178 (0.009)	3.067 (0.004)	3.210 (0.112)
B13	2.174 (0.055)	2.525 (0.014)	2.233 (0.010)	2.110 (0.003)	2.230 (0.027)
B14	3.732 (0.112)	3.462 (0.033)	2.994 (0.084)	2.991 (0.093)	3.054 (0.071)
B15	1.941 (0.068)	2.453 (0.102)	1.925 (0.004)	1.882 (0.019)	2.025 (0.062)
B16	1.968 (0.070)	2.143 (0.007)	1.725 (0.054)	1.902 (0.034)	2.064 (0.015)
B17	2.207 (0.022)	2.705 (0.059)	2.254 (0.016)	2.299 (0.021)	2.315 (0.039)
B18	3.317 (0.150)	3.094 (0.017)	2.912 (0.027)	2.939 (0.006)	3.175 (0.063)
B19	2.624 (0.030)	2.725 (0.065)	2.362 (0.006)	2.336 (0.122)	2.316 (0.037)
B20	2.555 (0.024)	2.620 (0.051)	3.002 (0.058)	2.893 (0.006)	2.258 (0.112)
STp	1.854 (0.162)	2.284 (0.144)	2.318 (0.065)	2.446 (0.013)	2.569 (0.010)

Values are absorbance measurements expressed as mean (SEM) of serum IgG levels against each bacterial peptide in healthy controls (HC), and active ulcerative colitis (aUC), quiescent ulcerative colitis (qUC), active Crohńs disease (aCD) and quiescent Crohńs disease (qCD) patients.

Table 4
Human serum IgA levels towards bacterial peptides.

Peptide	HC	aUC	qUC	aCD	qCD
B1	2.559 (0.014)	1.793 (0.010)	1.762 (0.081)	1.600 (0.015)	2.093 (0.001)
B2	2.650 (0.047)	1.611 (0.008)	1.953 (0.022)	1.481 (0.017)	1.968 (0.041)
B3	2.664 (0.026)	1.553 (0.013)	1.389 (0.024)	1.393 (0.001)	1.779 (0.048)
B4	2.986 (0.025)	2.072 (0.018)	1.890 (0.101)	1.657 (0.005)	2.148 (0.019)
B5	2.409 (0.095)	1.597 (0.009)	1.646 (0.042)	1.396 (0.001)	1.939 (0.038)
B6	2.318 (0.041)	1.581 (0.055)	1.487 (0.019)	1.282 (0.006)	1.777 (0.029)
B7	2.868 (0.002)	2.037 (0.036)	1.840 (0.067)	1.833 (0.053)	2.216 (0.026)
B8	2.592 (0.041)	1.749 (0.036)	1.719 (0.085)	1.568 (0.003)	2.043 (0.045)
B9	2.451 (0.011)	1.849 (0.008)	1.718 (0.119)	1.485 (0.036)	1.853 (0.049)
B10	2.625 (0.105)	1.858 (0.019)	1.796 (0.049)	1.734 (0.014)	2.396 (0.016)
B11	2.807 (0.046)	1.803 (0.006)	1.677 (0.085)	1.543 (0.024)	1.944 (0.004)
B12	2.977 (0.020)	2.091 (0.024)	2.033 (0.032)	1.919 (0.019)	2.469 (0.024)
B13	2.859 (0.011)	1.914 (0.009)	1.793 (0.062)	1.609 (0.013)	2.089 (0.031)
B14	3.148 (0.043)	2.241 (0.023)	1.732 (0.011)	1.718 (0.023)	2.653 (0.064)
B15	2.858 (0.075)	1.927 (0.070)	1.575 (0.050)	1.626 (0.030)	2.058 (0.044)
B16	3.571 (0.249)	2.390 (0.025)	2.216 (0.046)	2.140 (0.001)	2.690 (0.040)
B17	2.349 (0.226)	1.703 (0.054)	1.459 (0.026)	1.525 (0.036)	1.858 (0.065)
B18	3.075 (0.114)	2.013 (0.011)	2.015 (0.028)	1.997 (0.022)	2.682 (0.098)
B19	2.956 (0.012)	2.954 (0.069)	1.515 (0.059)	1.483 (0.023)	1.949 (0.108)
B20	2.934 (0.051)	1.816 (0.004)	1.879 (0.014)	1.794 (0.022)	2.240 (0.065)
STp	2.984 (0.094)	1.975 (0.023)	1.995 (0.060)	1.834 (0.063)	2.553 (0.063)

Values are absorbance measurements expressed as mean (SEM) of serum IgA levels against each bacterial peptide in healthy controls (HC), and active ulcerative colitis (aUC), quiescent ulcerative colitis (qUC), active Crohńs disease (aCD) and quiescent Crohńs disease (qCD) patients.

Table 5
Statistical differences of human serum IgA levels towards bacterial peptides.

Peptide	HC *vs* aUC	HC *vs* qUC	HC *vs* aCD	HC *vs* qCD	aUC *vs* qUC	aCD *vs* qCD	aUC *vs* aCD	qUC *vs* qCD
B1	****	****	****	****	ns	****	*	***
B2	****	****	****	****	****	****	ns	ns
B3	****	****	****	****	*	****	*	****
B4	****	****	****	****	ns	***	**	*
B5	****	****	****	***	ns	***	ns	*
B6	****	****	****	****	ns	****	***	***
B7	****	****	****	****	ns	**	ns	**
B8	****	****	****	****	ns	****	*	***
B9	****	****	****	***	ns	**	**	ns
B10	****	****	****	*	ns	****	ns	****
B11	****	****	****	****	ns	***	**	**
B12	****	****	****	****	ns	****	ns	***
B13	****	****	****	****	ns	****	****	***
B14	****	****	****	***	***	****	***	****
B15	****	****	****	****	**	***	**	***
B16	****	****	****	***	ns	*	ns	*
B17	**	***	***	*	ns	ns	ns	ns
B18	****	****	****	*	ns	**	ns	**
B19	ns	****	****	****	****	***	****	***
B20	****	****	****	****	ns	**	ns	*
STp	****	****	****	**	ns	****	ns	***

One-way ANOVA with Tukey correction was applied for each peptide to compare groups (HC: healthy controls, aUC: active ulcerative colitis, qUC: quiescent ulcerative colitis, aCD: active Crohńs disease, qCD: quiescent Crohńs disease), and p-values < 0.05 were considered significant (* < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001).

were different among all the study groups.

In the healthy gut, immune responses towards microbiota are predominantly driven by the secretory IgA (Zitomersky, Coyne, & Comstock, 2011). This fact might explain why the biomarker potential of the peptides was related with the IgA isotype as IBD patients displayed lower levels than HC, which might correspond to microbial gut alterations such as dysbiosis and lower microbiota diversity, driving therefore to lower IgA levels compared to the healthy mucosa. Although some investigations have been carried out to identify IBD biomarkers being clinical faecal calprotectin and serum C-reactive protein among the well-characterized ones, intense efforts in this field, still in its infancy, have been encouraged (Sands, 2015). Therefore, our screening with 20 new bacterial peptides might set the basis for further studies that explore and validate on larger and independent cohorts novel non-invasive IBD biomarkers.

3.2. Microbial peptides as novel immunomodulatory compounds on the human intestinal lamina propria

Some specific strains of probiotic bacteria might drive inflammatory activities in IBD, while certain lactic acid bacteria, the commensal *F. prausnitzii,* or bacterial metabolic products, among others, can mitigate inflammation. This is the case, for example, of the TNF-α down-regulation produced by *Lactobacillus casei* DN-114001 and *Lactobacillus bulgaricus* LB10 in *ex vivo* culture of inflamed ileum from CD patients (Borruel et al., 2002), and the reported ability of the former probiotic strain to counteract the pro-inflammatory influence exerted by *E. coli* on CD inflamed mucosa (Llopis et al., 2009). Hence, having proved the presence of different circulating levels of IgA directed towards the peptides in IBD patients compared with HC, we next aimed to study whether these 20 novel peptides also have the capacity to modulate the human intestinal cytokine milieu.

All bacterial peptides, excluding B2, modulated the intestinal cytokine profile on resting conditions (Supporting Information Fig. S1). Nevertheless, peptide B2, together with B3, B7, B8, B15, B17 and B18, modulated the cytokine profile in the presence of LPS (Supporting Information Fig. S2). These results confirm that all studied peptides are bioactive as they display immunomodulatory effects on the human intestinal mucosa. Indeed, all peptides, excluding B2, expanded the intestinal secretion of regulatory IL-10, a cytokine which plays a key role in the maintenance of intestinal homeostasis preventing inflammatory and autoimmune disorders (Iyer & Cheng, 2012). Together, and based on the global cytokine profiles elicited over LPMC either on resting condition or LPS, the bacterial peptides were classified into (i) tolerogenic; (ii) pro-inflammatory; and (iii) those with a mixed profile.

The group of tolerogenic peptides included peptides B4, B5, B9, B11 and B13 as they specifically expanded human intestinal levels of IL-10 on resting conditions (Fig. 1A) without exerting significant changes on the LPS-challenged cytokine milieu (Fig. 1B). This group also included peptide B7 which, in addition to expand IL-10 in the intestinal mucosa, also decreased the levels of pro-inflammatory adaptive immune mediators IL-17A and IL-8.

The group of pro-inflammatory peptides included peptides B2, B3,

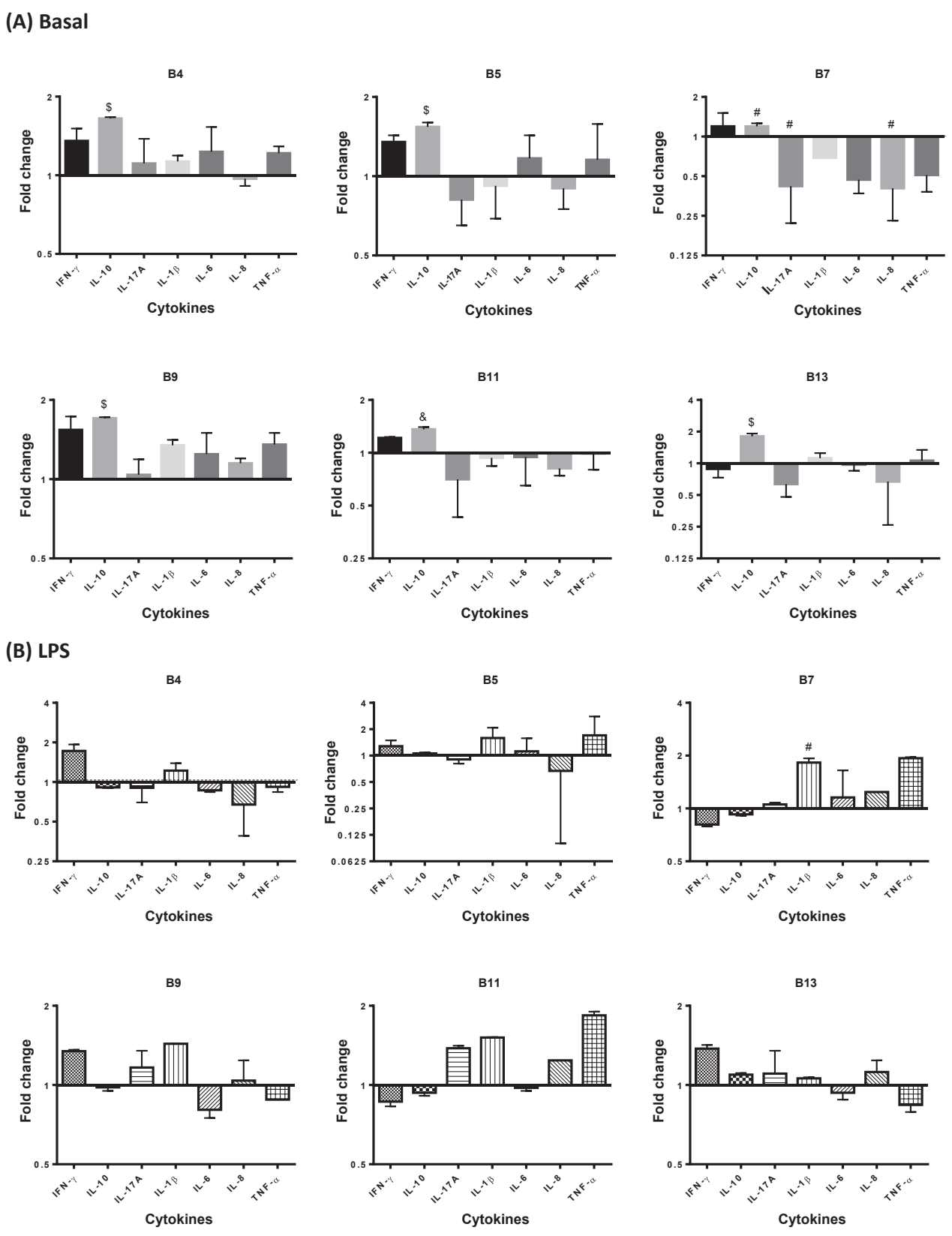

Fig. 1. Cytokine levels in LPMC supernatants following overnight culture with bacterial peptides B4, B5, B7, B9, B11 and B13. Results were represented as ratios (mean ± SEM) referred to (A) basal and (B) LPS conditions. One-way ANOVA with subsequent uncorrected Fisher's LSD test was applied to determine statistical differences for each cytokine *versus* basal and LPS, and p-values < 0.05 were considered significant (# < 0.05, & < 0.001, $ < 0.0001).

B15, B17 and B18. Hence, and although peptide B2 did not alter the cytokine milieu on resting conditions (Fig. 2A), it exacerbated the pro-inflammatory effect elicited by LPS as it further expanded IFN-γ at the time that decreased IL-10 (Fig. 2B). In a similar manner, peptide B3 primed a pro-inflammatory profile on the human mucosa expanding the secretion of Th1 cytokines (IFN-γ and TNF-α), while diminishing

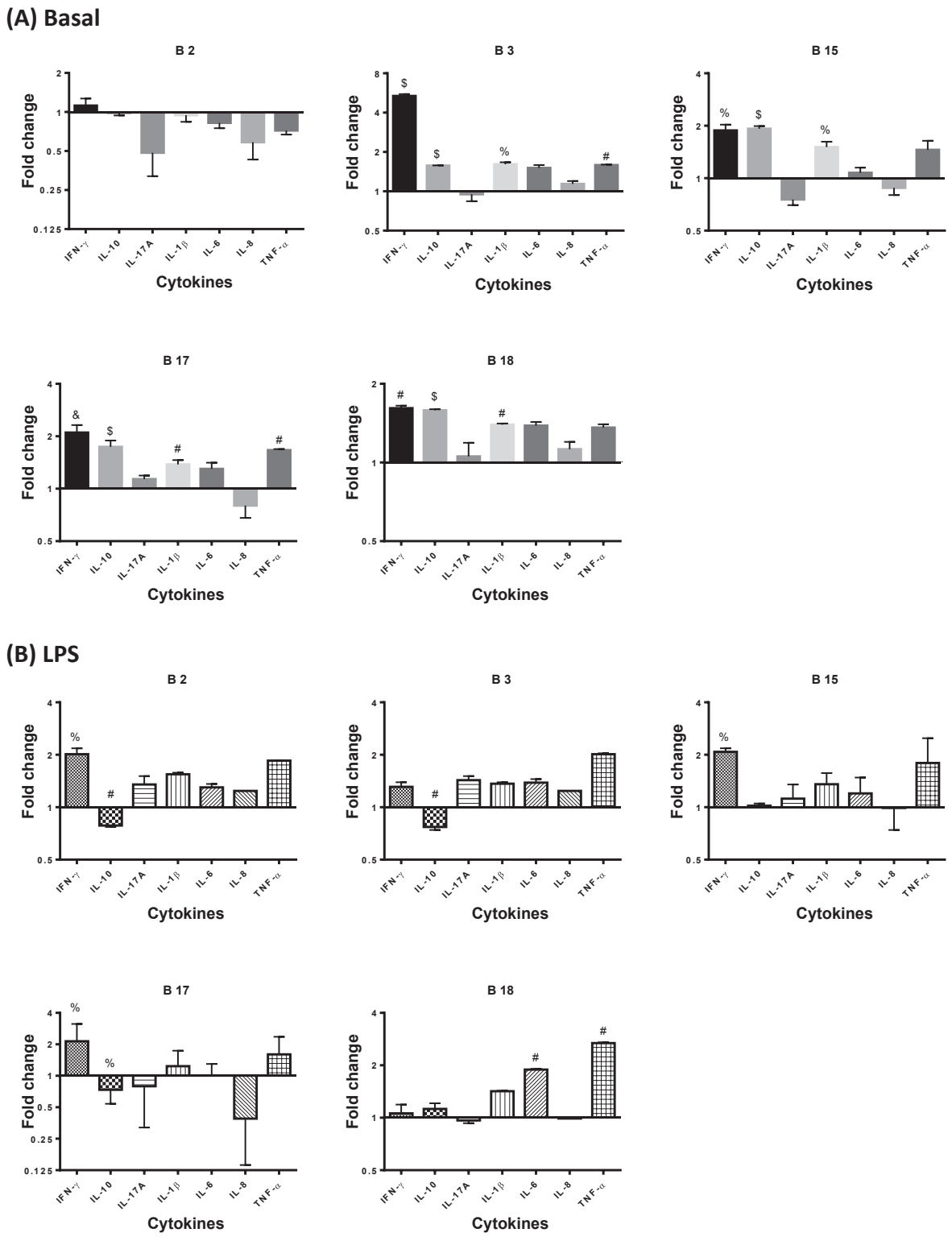

Fig. 2. Cytokine levels in LPMC supernatants following overnight culture with bacterial peptides B2, B3, B15, B17 and B18. Results were represented as ratios (mean ± SEM) referred to (A) basal and (B) LPS conditions. One-way ANOVA with subsequent uncorrected Fisher's LSD test was applied to determine statistical differences for each cytokine *versus* basal and LPS, and p-values < 0.05 were considered significant (# < 0.05, % < 0.01, & < 0.001, \$ < 0.0001).

secretion of IL-10 secretion in presence of LPS. Finally, and although peptides B15, B17 and B18 also expanded IL-10 in resting conditions, they also increased secretion of pro-inflammatory cytokines like IFN-γ and IL-1β, but also TNF-α in the case of B17 (Fig. 2A). Moreover, these pro-inflammatory effects were further exacerbated in the presence of LPS hence confirming their pro-inflammatory effect (Fig. 2B).

The last group included those peptides with a mixed profile, covering peptides B1, B6, B8, B10, B12, B14, B16, B19, B20 and STp. These peptides were categorized into this group given that, while they all expanded IL-10 secretion, they also expanded the secretion of other pro-inflammatory cytokines (Fig. 3A) although, as opposed to the previous group, they did not exacerbate the pro-inflammatory cytokine

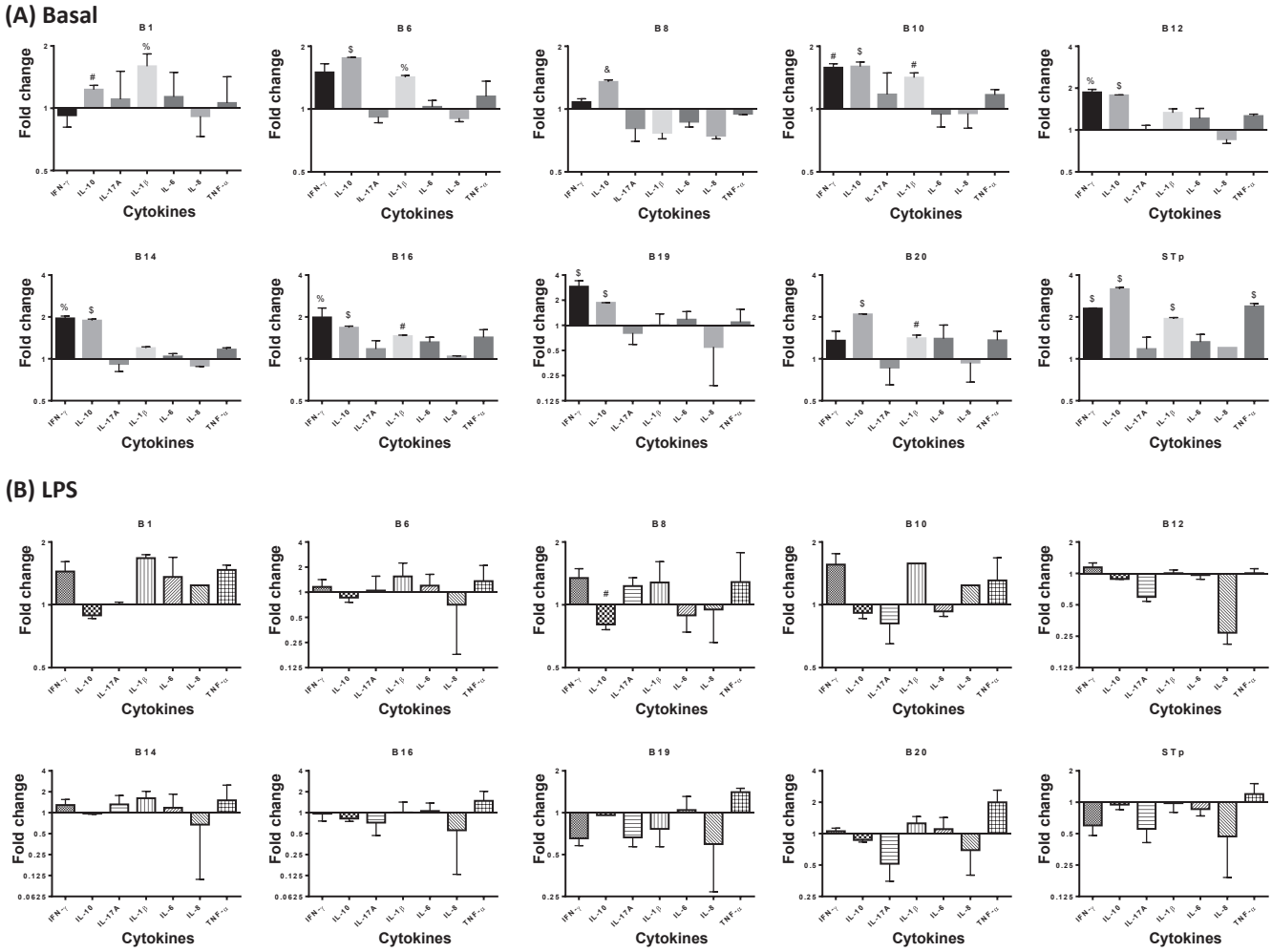

Fig. 3. Cytokine levels in LPMC supernatants following overnight culture with bacterial peptides B1, B6, B8, B10, B12, B14, B16, B19, B20 and STp. Results were represented as ratios (mean ± SEM) referred to (A) basal and (B) LPS conditions. One-way ANOVA with subsequent uncorrected Fisheŕs LSD test was applied to determine statistical differences for each cytokine *versus* basal and LPS, and p-values < 0.05 were considered significant (# < 0.05, % < 0.01, & < 0.001, $ < 0.0001).

milieu in the presence of LPS (Fig. 3B). Finally, peptide B8 was also included into this group given its unique capacity to modulate IL-10 both in resting conditions, where it expanded its secretion, but also in the presence of LPS where it diminished it. Despite all these peptides elicited a mixed profile on the human gastrointestinal mucosa, the anti-inflammatory STp was also classified into this category. Considering the restoration properties proved for STp on the colonic mucosa from UC patients (Al-Hassi et al., 2014; Bernardo et al., 2012), this issue deserves further experimentation not just in other gastrointestinal cellular models (Noben et al., 2017) but also in human IBD mucosa.

Although the use of probiotics has been suggested as complementary treatment for IBD patients, Tsilingiri et al. (2012) demonstrated that normally innocuous probiotic strains might be detrimental on inflamed IBD tissues. Hence, in the acute phase of the inflammatory disease where the epithelial integrity is compromised, the bacteria may get direct access to the lamina propria and elicits its detrimental effect. This fact, together with some findings where probiotic effects are not associated to the bacteria but with soluble metabolites released in the culture supernatant, made these authors to postulate these bacteria-related compounds as a safer alternative to the use of whole bacteria (Tsilingiri et al., 2012). Among the range of molecular effectors derived from probiotic or gut bacteria cell wall components, exopolysaccharides, secreted metabolites such as SCFA, conjugated linoleic acid or bacteriocins, and extracellular proteins and bioactive peptides have been reported (Ruiz et al., 2014). Regarding the study of microbial secreted peptides, bacterial peptides FR-16 and LR-17 (Hidalgo-Cantabrana et al., 2017), which correspond respectively to peptides B6 and B12 of our study, were demonstrated as the highest immunoactive ones within a set of 15 bacterial peptides evaluated on human PBMC in a way related to Th17 and Th22 responses. Moreover, peptide LR-17 (B12 on this study) stimulated GM-CSF and IL-1β cytokines, proposing a mechanism related to macrophage activation. Nevertheless, in our study in LPMC, those peptides did not alter the levels of IL-6, IL-17A and TNF-α as previously reported on PBMC, and only peptide B6 (FR-16) expanded the basal production of IL-1β. In regards to these different effects, some studies have tried to elucidate the structure-function relationship underlying the functionality of bioactive peptides (Li & Yu, 2015; Udenigwe, 2014). For instance, novel milk casein-derived peptides with mucin stimulatory activity in intestinal HT29-MTX cells, all containing the N-terminus tripeptide Tyr-Phe-Tyr, were recently evaluated by molecular dynamics simulations with the μ-opioid intestinal receptor and demonstrated to differently interact with the active binding site, decreasing activity depending on the amino acid composition at the C-terminus (Fernández-Tomé et al., 2016). A hydrophobic nature has been demonstrated to facilitate the immunomodulatory activity of food peptides (Chalamaiah, Yu, & Wu, 2018). Indeed, peptide B12 showing the highest percentage of hydrophobic amino acids (Supporting Information Table S1), stimulated intestinal IL-10 and IFN-

γ cytokines in our study and was identified as an immunoactive peptide (LR-17) on PBMC (Hidalgo-Cantabrana et al., 2017). On the other hand, immune responses elicited by bacterial peptides seem to be dependent on the *in vitro* model used, presenting marked differences between systemic PBMC and intestinal LPMC from human healthy donors. Therefore, the screening of gastrointestinal tract-related bioactive peptides should be performed using models of the intestinal mucosa which may include intestinal cell lines (Fernández-Tomé, Sanchón, Recio, & Hernández-Ledesma, 2018) or, alternatively, the use of real intestinal colonic samples as we have performed here. Moreover, additional structure-activity and mechanistic studies with peptides of interest are needed and currently ongoing to further elucidate their immunomodulatory potential in the context of IBD.

Finally, and specifically focused on the set of peptides selected for our study, peptide B7 from *Bifidobacterium longum* subsp. *longum* displayed the most promising tolerogenic effects on the human intestinal mucosa by inducing IL-10 and diminishing IL-17A and IL-8 in resting conditions, while activating the innate immune IL-1β response together with LPS. Accordingly, this probiotic bacterium has been shown to ameliorate chemical-induced colitis in mice by suppressing IL-17A response and restoring the Th17/Treg balance (Lim, Jeong, Jang, Han, & Kim, 2016; Miyauchi et al., 2013). Our findings thus contribute to the role of bacterial peptides and metabolites as regulatory molecular players for host immune-mediated disorders (Rooks & Garrett, 2016); and in the framework of IBD, to studies evaluating the properties of bioactive peptides to stimulate tolerogenic intestinal dendritic cells (Al-Hassi et al., 2014), induce Treg responses (Lopez, Gonzalez-Rodriguez, Gueimonde, Margolles, & Suarez, 2011) and perform immunosuppressive and anti-inflammatory activities (Lightfoot et al., 2015) in IBD patients.

4. Conclusions

Elucidation of host/microbiota interplay within the intestinal mucosa represents a novel line of attack in the approach of IBD. Our experiments showed that serum IgA and, to a lower extent, IgG levels developed against bacterial peptides discriminated HC from IBD patients. Moreover, IgA serum levels might discriminate UC and CD patients, but also, within each condition, patients with endoscopically active or quiescent disease. This highlights the need of further studies on circulating antibodies raised towards intestinal microbial peptides as novel non-invasive biomarkers to aid on IBD diagnosis and monitoring. Furthermore, all bacterial peptides assessed in this study demonstrated bioactive functionality at the human intestinal mucosa given their capacity to modulate the intestinal cytokine milieu, highlighting a novel secreted peptide from *Bifidobacterium longum* subsp. *longum* as the most promising tolerogenic peptide. Altogether, our results not only contribute to the evidence of the microbiota/immune system crosstalk by soluble factors, but also propose bacterial peptides encrypted in the human protein microbiome as potential biomarkers with further functionality in the immunomodulation of gut homeostasis.

Ethics approval

Ethics approval to conduct this research was obtained by the Ethics Committee at Hospital Universitario de La Princesa in Madrid (Spain).

Author contributions

Study concept and design was performed by BS, DB and JPG. Peptide identification and selection was done by BS. ELISA experiments were performed by AMA, ADG, and JMGR. Processing of intestinal samples and culture was performed by SFT, AMA, ACM, IMG and LOM. Multiplex experiment was performed by AMA. Patients identification, recruitment, and biological samples obtention was performed by CS, MC and JPG. Data analysis and interpretation was performed by SFT, AMA and DB. Funds required to perform this study were obtained by JPG and DB. The manuscript was drafted by SFT and further edited by MC, JPG, BS and DB. All authors approved the final version of the submitted manuscript.

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Conflict of interest

Borja Sánchez is on the scientific board and is co-founder of Microviable Therapeutics SL. The other authors have declared no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2018.11.036.

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