UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II

DOTTORATO DI RICERCA IN

MEDICINA CLINICA E SPERIMENTALE

CURRICULUM IN SCIENZE PEDIATRICHE TRASLAZIONALI

XXXIV Ciclo

(Anni 2018-2021)

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TESI DI DOTTORATO

La gliadina induce l'attivazione del complesso di mTor eNFκ-β in cellule epiteliali intestinali:ruolo del probiotico Lactobacillus Paracasei CBA L74

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1 Introduzione

La malattia celiaca (CD) è una malattia autoimmune che colpisce principalmente l'intestino tenue [1], a causa della risposta anomala del sistema immunitario all'ingestione di glutine. Si verifica in soggetti con suscettibilità genetica (positività HLA-DQ2/DQ8 e geni non-HLA) e sotto l'influenza di trigger ambientali, tra cui, glutine, infezioni virali e disbiosi del microbiota intestinale [2]. Sebbene il 30-40% della popolazione mondiale sia portatore del genotipo HLA DQ2/DQ8, solo l'1-1,5% di essi esprime il fenotipo CD, il che significa che altri geni insieme ad altri fattori ambientali sono coinvolti in questa malattia [2,3]. L'infiammazione nella CD include un aumento della conta dei linfociti intraepiteliali (IEL) [4,5]. Altre caratteristiche della CD sono la presenza di una risposta adattativa mediata dalle cellule T al glutine [6,7] e di anticorpi endomisiali specifici (EMA, chiamati anche AEA), anticorpi anti-tessuto transglutaminasi (TTG, a-tTG, TTA), e/o anticorpi antigliadina deamidata (DGP) che svolgono un ruolo importante nel work-up sierologico della CD. Questi anticorpi supportano fortemente la diagnosi di CD. Nei bambini, la biopsia intestinale è necessaria quando il titolo anticorpale è basso e i sintomi sono presenti. Nei pazienti pediatrici con tTG-IgA elevato, l'ESPGHAN raccomanda che la decisione di eseguire o meno biopsie duodenali sia presa durante un processo decisionale condiviso tra il gastroenterologo pediatrico/specialista della celiachia, i genitori/accompagnatori; tale biopsia deve essere eseguita quando i pazienti seguono ancora una dieta contenente glutine [8]. La diagnosi è confermata dal riscontro di un'atrofia intestinale con accorciamento dei villi intestinali e aumento della proliferazione delle cripte. Il glutine è scarsamente digerito nell'intestino umano con o senza CD. Ed è proprio questo il fattore ambientale responsabile della malattia, il glutine, una proteina del grano ed in particolare la gliadina. Di tutta la gliadina solo due peptidi restano non digeriti [4-5]: il 33-mer (P55-87) e il 25-mer (P31-55). Questi due peptidi sono i peptidi principali che sono attivi in vivo nell’intestino celiaco dopo

l'ingestione di glutine. I peptidi di glutine non digeriti attraversano intatti la mucosa dell'intestino tenue. Nella sottomucosa dell'intestino tenue l'enzima umano transglutaminasi tissutale (tTG) deamida i peptidi di glutine, portando ad un aumento dell'affinità di legame con le molecole dell'antigene leucocitario umano (HLA) DQ2 e HLA DQ8, scatenando successivamente una reazione infiammatoria nei pazienti con CD [9]. L'unico trattamento attualmente disponibile nella CD è una dieta senza glutine per tutta la vita. Recentemente è stato descritto un ruolo per il microbiota intestinale nella CD. Ci sono diversi studi trasversali che confrontano il microbiota nei pazienti e nei controlli e che descrivono una disbiosi nei pazienti con CD prima e dopo la dieta senza glutine. Negli ultimi anni il mondo scientifico si è concentrato molto sul ruolo dei pro- pre e post-biotici su diverse malattie che possono colpire l'uomo, tra cui la CD.

1.1 Pro-pre e post-biotici

I pro-biotici sono microrganismi vivi, la maggior parte dei quali sono batteri simili e/o uguali a batteri benefici che già si trovano naturalmente nell'intestino umano. **La definizione internazionalmente accettata di "probiotico" è quella elaborata nel 2001 [10] dalla FAO e dall'OMS: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host". In Italia il Ministero della Salute [11] ha definito i probiotici "microrganismi che si dimostrano in grado, una volta ingeriti in adeguate quantità, di esercitare funzioni benefiche per l'organismo" sostanzialmente riprendendo la definizione delle due organizzazioni ONU.** I probiotici sono stati ampiamente studiati in una varietà di malattie gastrointestinali. I probiotici hanno un ruolo importante nel mantenimento dell'equilibrio immunologico nel tratto gastrointestinale attraverso l'interazione diretta con le cellule immunitarie.

Questi microrganismi sono capaci di metabolizzare le fibre alimentari, che prendono il nome di pre-biotici. Queste fungono da nutrimento per i probiotici aumentandone il numero. Sono

definite come "componenti alimentari non assorbibili che stimolano beneficamente uno o più gruppi di ‘’microbi amici’’ dell'intestino e quindi hanno un effetto positivo sulla salute umana" [12]. Naturalmente presenti negli alimenti, come carciofi, aglio, cipolle e altri. Può essere necessario consumare grandi quantità di questi alimenti per avere un effetto "bifidogenico". Per questo motivo, è più facile prendere un integratore pre-biotico o una combinazione di integratori pro-biotici e pre-biotici (simbiotici) per ottenere livelli ottimali. Sono, inoltre, resistenti all'idrolisi da parte degli enzimi digestivi e non vengono assorbiti nella parte superiore del tratto gastrointestinale. Quando raggiungono l'intestino crasso, sede del microbiota, stimolano la crescita di alcuni microrganismi [12]. Dal metabolismo di questi polisaccaridi si generano acidi grassi a catena corta (SCFA), tra cui acetato, propionato e butirrato, i quali rientrano nella categoria dei post-biotici.

I post-biotici sono noti come "probiotici non vitali", "probiotici inattivati" o "probiotici fantasma" e si riferiscono sia a cellule microbiche non vitali che a fattori solubili secreti da batteri vivi o rilasciati dopo la loro lisi, compresi vari componenti della superficie cellulare, acido lattico, acidi grassi a catena corta (SCFA) e peptidi bioattivi tra gli altri metaboliti. Quando somministrati in quantità sufficienti, questi possono contribuire al miglioramento della salute dell'ospite, anche se i meccanismi esatti non sono ancora noti. L'inattivazione batterica avviene di solito tramite un trattamento termico leggero. I vantaggi dell'uso dei post-biotici includono la loro maggiore stabilità, in quanto non contengono batteri vivi, e la loro maggiore sicurezza rispetto ai pro-biotici, in quanto riducono il rischio di traslocazione microbica, di infezione o di aumento delle risposte infiammatorie nei consumatori con sistemi immunitari squilibrati o compromessi.

1.2 Il microbiota è alterato nella CD

Il microbiota è una comunità ecologica di microrganismi, si concentra nel tratto intestinale e viene rapidamente alterato da fattori esterni. Esiste poi il microbioma che rappresenta il

genoma collettivo di tutti i microrganismi di una data nicchia ambientale. Cambiamenti nel microbiota e di conseguenza nel microbioma, hanno un impatto sull'omeostasi di tutto il corpo. La composizione del microbiota intestinale dipende da molti fattori, come l'età, la posizione geografica, la dieta, la genetica, il parto naturale e l'allattamento; quindi è impossibile avere lo stesso microbiota in tutti i soggetti così come è impossibile definire il microbiota ideale. Il microbiota gioca un ruolo fondamentale nell'induzione, formazione e funzionamento del sistema immunitario dell'ospite quindi la sua alterazione è generalmente associata alla risposta del sistema immunitario e all'infiammazione [13]. Nella CD sono state trovate alterazioni del microbiota ed è interessante notare che il microbiota dei pazienti con CD può cambiare nella diversa fase di la malattia. Ci sono "firme del microbiota" differenti:

a) I pazienti nella fase acuta della malattia a GCD (Gluten Containing Diet) presentano un'alterazione del microbioma intestinale con aumentando di E. coli, ML615J-28, Slackia, Victivallaceae, Enterobacteriaceae, Clostridiaceae, Coriobacteriaceae e specie non classificate di Clostridiales e Lachnospriraceae, in diminuzione C. lituseburense, Lactobacillus, F. Prausnitzii, Bifidobacterium, Dorea, B. wexlerae, Lachnospriraceae, A. hadrus, E. hallii, Veillonellaceae, R. bromi, R. faecis.

In questi pazienti i batteri che hanno un effetto protettivo come Bifidobatteri, Firmicutes, Lactobacilli e Streptococchi sono più bassi rispetto ai controlli sani. I pazienti con CD attiva hanno un aumento di batteri gram-negativi come Bacteriodes, Bacterioidetesfragilis, Prevotella, E. coli, Proteobatteri, Haemophilus, Serratia, Klesbisella [14,15]. Nel microbiota intestinale dei pazienti con CD, la presenza di batteri patogeni come Clostridium perfringens e C. difficile può essere una conseguenza della riduzione dei Bifidiobatteri e apparentementesembrano promuovere il rischio di sviluppare la malattia celiaca in paziente a rischio [16].

b) I pazienti con CD a GFD (Gluten Free Diet) per almeno 2 anni, hanno mostrato una composizione del microbiota fecale simile ai controlli sani caratterizzati da una ridotta diversità di Lactobacillus e Bifidobacterium, indicando che GFD potrebbe normalizzare la composizione del microbiota intestinale in CD [17]. I dati sono dipendenti dal tempo della GFD, suggerendo che una GFD prolungata potrebbe modificare la composizione del microbiota.

c) La GFD nei soggetti sani influenza il microbiota intestinale riducendo il Bifidobacterium, Clostridium lituseburense e Faecali bacterium praunsnitzii e aumentando l'Enterobacteriaceae e Escherichia coli [18]. Inoltre, utilizzando una dieta a basso contenuto di glutine, è possibile osservare un aumento delle specie non classificate di Clostridiales.

La GFD influenza il microbiota intestinale dei soggetti sani aumentando E. Coli, ML615J-28, Slackia, Victivallaceae,Enterobacteriaceae, Clostridiaceae, Coriobacteriaceae e specie non classificate di Clostridiales e Lachnospriraceae, diminuendo C. lituseburense, Lactobacillus, F. prausnitzii, Bifidobacterium, Dorea, B. wexlerae, Lachnospriraceae, A. hadrus, E. hallii, Veillonellaceae, R. bromi, R. faecis.

In conclusione, i pazienti con CD presentano un microbiota diverso rispetto ai soggetti normali nella fase acuta della malattia, mangiando glutine. La GFD può modulare il microbiota intestinale di pazienti con CD rispetto ai soggetti sani, e può indurre alcune alterazioni nel microbiota in soggetti sani a GFD. La maggior parte degli studi sul microbiota intestinale in CD sono descrittivi, da questi studi è difficile determinare se l'insorgenza della CD sia dovuta all'alterazione del microbiota o se questo è secondario al danno intestinale presente nella CD. In questo contesto, i pre-probiotici hanno le caratteristiche per essere utili nel prevenire o anche migliorare la disbiosi nella CD. I post-biotici non sono ancora stati testati nella CD, ma dai dati in vitro sembrano essere in grado di prevenire alcuni effetti della gliadina e dei peptidi di gliadina.

1.3 Peptide tossico della gliadina: P31-43

Il danno intestinale nella celiachia consiste nell' infiammazione e nel rimodellamento della mucosa, con appiattimento dei villi ed ipertrofia delle cripte. Vi sono due tipi principali di risposta infiammatoria ai peptidi del glutine nella celiachia [19]: vi è la risposta adattativa mediata da cellule T CD4+ ad alcuni peptidi: prototipo è il 33 mer della A-Gliadina, resistente alla digestione gastrica, intestinale endoluminale e parietale. Il peptide viene deamidato ad opera della trasglutaminasi tissutale (tTG) e presentato dagli antigeni di istocompatibilità di classe II, DQ2 e DQ8, alle cellule T CD4+, con risposta del tipo Th1, mediata da gamma interferone e altre citochine proinfiammatorie. Vi è poi la risposta, non mediata da cellule T, ad altri peptidi: prototipo il peptide 31-43 della A-gliadina (P 31-43). Il P 31-43, anche esso resistente alla digestione gastrica ed intestinale, provoca infiammazione con meccanismi molteplici, il più noto dei quali consiste in una risposta da stress/innata e proliferativa, mediata da EGF e IL15. Il peptide P31-43 è anche un fattore di crescita per varie linee cellulari e per l'enterocita del celiaco, in quanto è in grado di attivare il sistema EGF-EGFR [20] il più potente mitogeno presente nel nostro organismo. La proliferazione degli enterociti delle cripte del celiaco indotta da gliadina (P31-43) è non solo EGF dipendente, ma anche IL15-dipendente [21]. La proliferazione degli enterociti delle cripte e la risposta immune innata alla gliadina del celiaco, sono regolate da una cooperazione tra EGF e IL15 [22]. Si viene così a delineare una duplice azione della gliadina nell'induzione del danno della mucosa dei soggetti celiaci. Da un lato la gliadina può attivare la risposta T-mediata e dall'altro può indurre una risposta innata, mediata dall'IL15, ed effetti proliferativi mediati dall'EGF. Gli effetti congiunti di questa duplice azione inducono la lesione tipica della mucosa celiaca. Resta da spiegare la maggiore suscettibilità del celiaco a queste particolari attività biologiche di alcuni peptidi della gliadina, in particolare del P31-43. Il traffico intracellulare delle vescicole di endocitosi regola l'endocitosi dell'EGFR e

degli altri recettori, e numerose funzioni cellulari e vie di signaling. Dopo il legame dell'EGF al suo recettore EGFR, e la attivazione di questo, il complesso EGF-EGFR è reclutato in vescicole di endocitosi ricoperte da clatrina e internalizzato negli endosomi precoci e poi negli endosomi tardivi e nel corpo multi vescicolare, per essere poi o riciclato sulla superficie cellulare oppure trasportato ai lisosomi, dove è degradato ed inattivato. Nelle cellule(enterocita e fibroblasto) del celiaco il traffico vescicolare di EGF è alterato. In particolare il traffico vescicolare del complesso EGF-EGFR è ritardato con accumulo di EGFR attivo negli endosomi precoci e attivazione del signaling a valle di EGFR, con conseguente aumento della proliferazione degli enterociti [22]. Queste alterazioni di base possono rappresentare, nei tessuti del celiaco una condizione predisponente all'azione dannosa di alcuni peptidi della gliadina, in particolare del P31-43. Infatti il P31-43 si localizza nelle cellule dei soggetti normali negli endosomi precoci e ritarda il traffico vescicolare probabilmente per interferenza con la localizzazione di Hrs sulla membrana degli endosomi (Hrs è una molecola chiave nella regolazione della maturazione degli endosomi) [21]. P31-43 riproducecosì nella cellula normale il fenotipo celiaco. Le stesse vie metaboliche influenzate dal P31-43 sarebbero già costitutivamente alterate nel celiaco, che risulterebbe pertanto più sensibileall'azione dannosa di questo peptide [22]. Ma il glutine e altre proteine del grano possono provocare danno (infiammazione) intestinale ed extra intestinale anche in soggetti non celiaci. Per esempio, nel topo, l'eliminazione del glutine dalla dieta riduce l'aumento di grasso, l'infiammazione dei tessuti e l'aumento della resistenza all'insulina provocati da eccesso di grassi nella dieta [23]. Inoltre una particolare frazione delle albumine di grano, quella che inibisce l'alfa amilasi e la tripsina, causa infiammazione intestinale per attivazione del TLR4 [24]. Il glutine stesso potrebbe rappresentare un fattore di rischio anche in patologie diverse dalla celiachia, per esempio nel diabete di tipo 1. È noto infatti che nei bambini con diabete insulinodipendente, non celiaci, vi sono segni di infiammazione dell'intestino [25-26] e possibili triggers di questa infiammazione sono virus e proteine

alimentari [27]. Più in particolare segni di alterata risposta immune mucosale alla gliadina sono stati evidenziati nel diabete di tipo 1 sia per challenge rettale in vivo con peptidi del glutine [28] che per challenge in vitro della mucosa dell'intestino tenue con gli stessi peptidi [29]. Nell'insieme queste osservazioni suggeriscono che i cereali contenenti glutine non sono sempre dei "buoni" alimenti. Sorge forse anche il quesito se i grani oggi utilizzati nell'alimentazione umana siano più dannosi di quelli ancestrali, che l'uomo iniziò a coltivare all'inizio del neolitico. È possibile che l'aumento dell'incidenza della malattia celiaca (e di altre intolleranze al glutine) sia da connettere all'aumento del consumo di glutine e alla qualità del glutine stesso presente nei cereali della nostra dieta. Probabilmente è da inquadrare in questo contesto la presunta intolleranza al glutine non celiaca (gluten sensitivity), che ha fatto sviluppare una vera e propria moda delle diete prive di glutine in molti paesi. Di fatto la letteratura scientifica su questo argomento è contraddittoria e confusa. Mentre da un lato sono state descritte condizioni patologiche che migliorano eliminando dalla dieta il glutine, dall'altra è stato dimostrato che i pazienti considerati "sensibili" al glutine sono in realtà sensibili ai FODMAPS, oligosaccaridi non digeribili e fermentabili nell'intestino, presenti nella dieta con glutine [30].

1.4 Il ruolo patogenico della chinasi m-TOR nella malattia celiaca

La proteina mTOR, un regolatore dell'attivazione e funzione di molteplici tipi cellulari, inclusi linfociti T e macrofagi, facilita la produzione di citochine infiammatorie coinvolte nella patogenesi di malattie immunitarie. Sulla base di tali dati è ipotizzabile che un'iperattivazione di mTOR possa contribuire all' amplificazione e perpetuazione della risposta infiammatoria in corso di malattia celiaca. Attraverso l'utilizzo di sofisticate tecniche di biologia cellulare e molecolare è stato possibile documentare una marcata attivazione di mTOR, associata ad una ridotta espressione dei suoi inibitori fisiologici, nella

mucosa duodenale di pazienti con celiachia attiva rispetto a quanto osservato nella mucosa duodenale dei controlli [31]. La **mTOR** (acronimo di **mammalian target of rapamycin**, *bersaglio della rapamicina nei mammiferi*) è una protein-chinasi che fosforila serina e treonina, regola la crescita, la proliferazione, la motilità e la sopravvivenza delle cellule, la sintesi proteica e la trascrizione. La proteina mTOR integra lo stimolo proveniente da percorsi superiori, inclusi insulina, fattori crescita (come IGF-1 e IGF-2) e mitogeni. La mTOR inoltre percepisce anche i nutrienti cellulari, i livelli di energia e lo stato redox. Il pathway di mTOR appare sregolato in diverse patologie umane, specialmente in alcuni tipi di cancro.

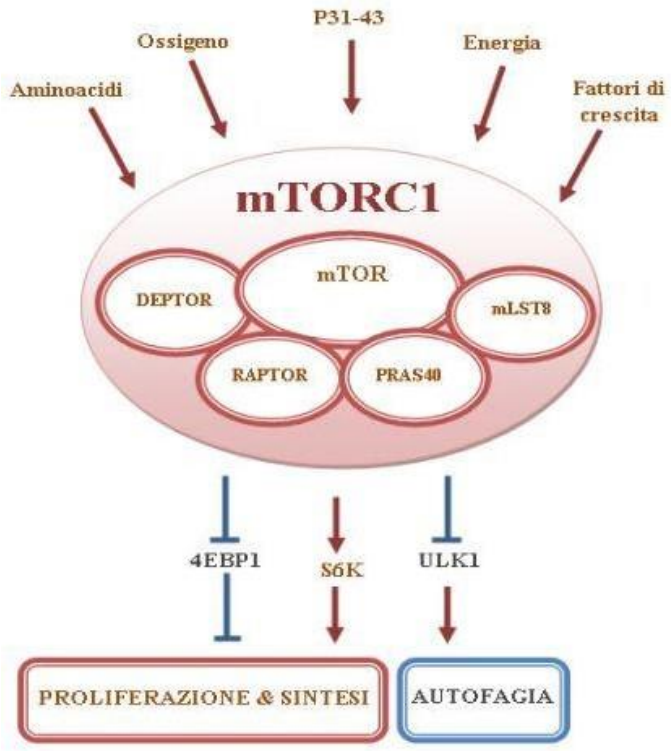


Fig.1 Attività del complesso mTORC1.

Questa chinasi è costituita da due strutture che funzionalmente sono diverse, mTOR Complex 1(figura 1) ed mTOR Complex 2. L'attività di m-TORC1 è stimolata da insulina, fattori di crescita, aminoacidi (in particolare la leucina) e stress ossidativo. mTORC1 è inibito da un basso livello di nutrienti, da una carenza di fattori di crescita, da stress reduttivo, dalla caffeina, dalla rapamicina, dall'acido farnesiltiosalicilico e dalla curcumina. I due bersagli di mTORC1 meglio caratterizzati sono la protein-chinasi 1 p70-S6 (S6K1) e 4E-BP1, ovvero la proteina che lega il fattore eucariotico di iniziazione 4E (eIF4E). M-TORC1 fosforila S6K1 su almeno due residui, causando per lo più la modificazione di un residuo di treonina (T389). Questo evento stimola la successiva fosforilazione di S6K1 ad opera di PDK1. La S6K1 attiva può ora stimolare l'inizio della sintesi proteica attraverso l'attivazione della proteina ribosomiale S6, un componente del ribosoma, e altri componenti dell'apparato trascrizionale. mTORC1 va anche a fosforilare 4E-BP; 4E-BP non fosforilato lega strettamente il fattore IF-4E, prevenendo il suo legame all' mRNA e di conseguenza il reclutamento del complesso di iniziazione ribosomiale. Al contrario se m-TORC1 fosforila E4-BP il fattore IF-4E viene rilasciato, e può andare a svolgere la sua funzione [32]. Quando tutti gli stimoli che attivano m-TORC1 sono bassi o assenti, m-TORC1 disattiva la sua funzione chinasica ed in forma inattiva stimola la formazione del complesso proautofagico contenente ULK1, chinasi la cui attivazione determina la formazione degli autofagosomi [33]. M-TORC2 fosforila la chinasi serina/treonina Akt/PKB sul residuo di serina S473. La fosforilazione di questa serina stimola la fosforilazione da parte di Akt su una treonina (T308) di PDK1 e comporta piena attivazione di Akt. Diversi studi suggeriscono che Akt regola l'attività trascrizionale del fattore NF-κB [34].

1.5 LC3, marker dell'autofagia

La regolazione del processo autofagico può essere mediata dal pathway di mTOR, che agisce da segnale inibitorio. La via di mTOR viene inattivata dalla mancanza di nutrienti, da bassi livelli energetici o da segnali di danno al DNA: in tutti questi casi è necessario innescare i meccanismi dell'autofagia. L'autofagia (figura 2.) è il più importante pathway cellulare deputato alla degradazione delle proteine e degli organelli citosolici. Il meccanismo autofagico ha un ruolo indispensabile nel mantenimento dell'omeostasi cellulare e nel turnover del materiale citoplasmatico [35]. Dal punto di vista molecolare, i pathways autofagici sono molteplici e complessi. Hanno un ruolo centrale le proteine ATG1-ATG35, prodotte dagli autophagy-related-genes: queste proteine formano complessi funzionali che mediano i principali steps autofagici.

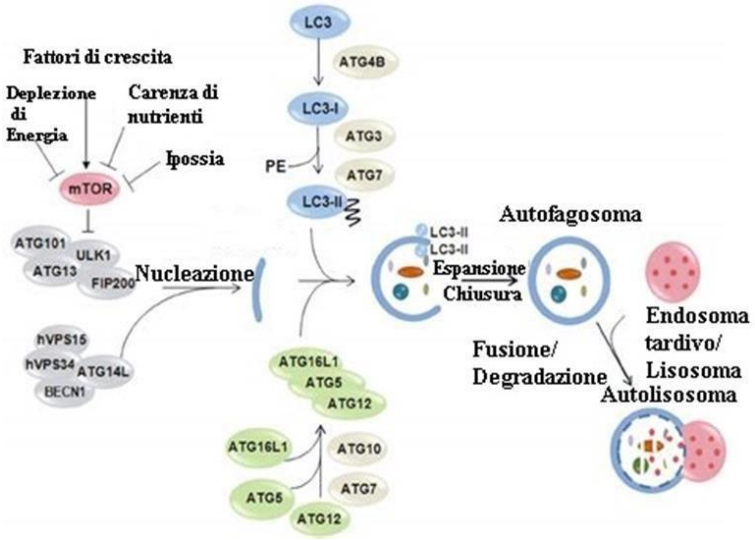


Fig.2 Pathway dell'autofagia.

1.6 NFK-β

Il fattore di trascrizione NF-κB regola molteplici aspetti delle funzioni immunitarie innate e adattative e funge da mediatore fondamentale delle risposte infiammatorie. NF-κB induce l'espressione di vari geni proinfiammatori, compresi quelli che codificano per citochine e chemochine, e partecipa anche alla regolazione dell'inflammasoma. Inoltre, NF-κB svolge un ruolo fondamentale nella regolazione della sopravvivenza, dell'attivazione e della differenziazione delle cellule immunitarie innate e delle cellule T infiammatorie. Di conseguenza, l'attivazione deregolata di NF-κB contribuisce ai processi patogenetici di varie malattie infiammatorie. Il fattore nucleare-κB (NF-κB) rappresenta una famiglia di fattori di trascrizione inducibili, che regola una vasta gamma di geni coinvolti in diversi processi delle risposte immunitarie e infiammatorie [36]. Questa famiglia è composta da cinque membri strutturalmente correlati, tra cui NF-κB1 (denominato anche p50), NF-κB2 (denominato anche p52), RelA (denominato anche p65), RelB e c-Rel [37]. L'attivazione di NF-κB coinvolge due principali vie di segnalazione, le vie canoniche e non canoniche (o alternative), entrambe importanti per la regolazione delle risposte immunitarie e infiammatorie nonostante le loro differenze nel meccanismo di segnalazione [38,39]. Il meccanismo principale per l'attivazione canonica di NF-κB è la degradazione inducibile di IκBα, innescata attraverso la sua fosforilazione sito-specifica da parte di un complesso IκB chinasi (IKK) multi-subunità [36,40]. IKK è composto da due subunità catalitiche, IKKα e IKKβ, e una subunità regolatoria denominata modulatore essenziale NF-κB (NEMO) o IKKγ [41]. L' IKK può essere attivato da diversi stimoli, tra cui citochine, fattori di crescita, mitogeni, componenti microbici e agenti di stress [42]. Dopo l'attivazione, IKK fosforila IκBα a due serine N-terminali e, quindi, innesca la degradazione IκBα ubiquitina-dipendente nel proteasoma, con conseguente traslocazione nucleare rapida e transitoria dei membri canonici NF-κB prevalentemente p50/RelA e p50/c- Rel [40,43,44].

NF-Kβ è costituito dalle proteine RelA e p50, e nella forma inattiva si trova nel citosol legato ad una proteina inibitoria IkBα; attraverso l'intermediazione dei recettori integrali della membrana, una varietà di segnali extracellulari può attivare l'enzima IκB kinase (IKK). L'IKK a sua volta fosforila la proteina IκBα portando alla sua ubiquitinazione e degradazione nel proteasoma. In questo modo NF-κB viene reso disponibile. L' NF-κB attivato è in seguito traslocato nel nucleo dove si lega a specifiche sequenze del DNA denominate ‘’*response elements’’* (RE). Il complesso DNA/NF-κB poi richiama altre proteine quali i coattivatori e l'RNA polimerasi che trascrive il DNA in mRNA, il quale, infine, è esportato nel citosol e tradotto in proteina (figura 3). Ciò porta ad un cambiamento delle funzioni della cellula, come ad esempio la produzione di citochine pro-infiammatorie.

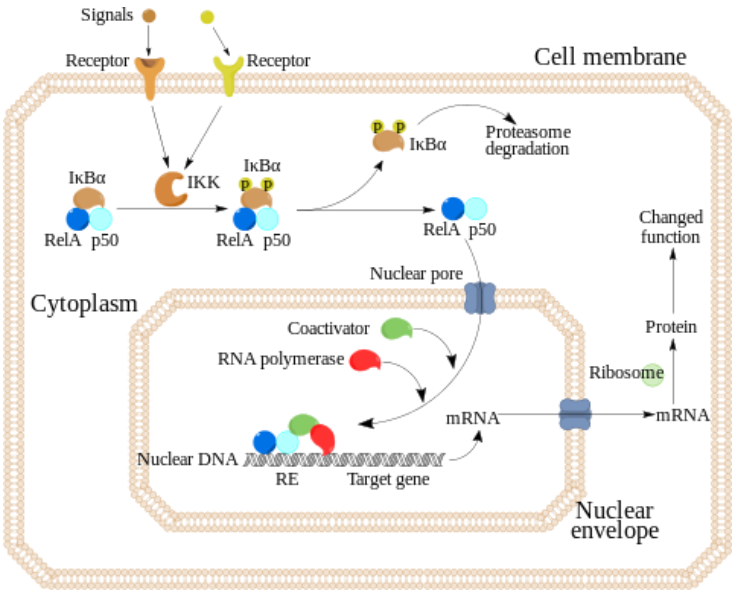


Fig.3 Attivazione pathway pNFκβ.

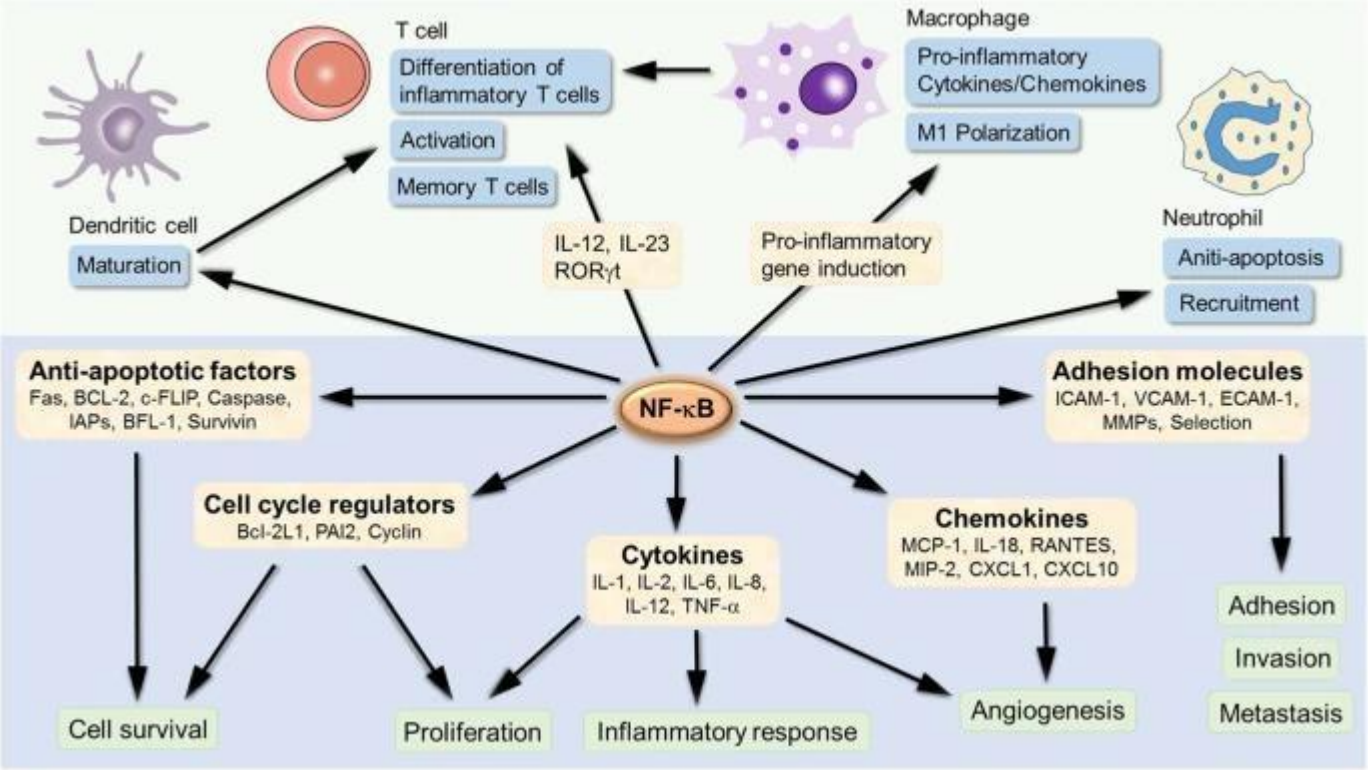


Figura 4.Geni bersaglio di NF-kB coinvolti nello sviluppo e nella progressione dell'infiammazione. NF-κB è un fattore di trascrizione inducibile. Dopo la sua attivazione, può attivare la trascrizione di vari geni e quindi regolare l'infiammazione. NF-κB prende di mira l'infiammazione non solo direttamente aumentando la produzione di citochine infiammatorie, chemochine e molecole di adesione, ma anche regolando la proliferazione cellulare, l'apoptosi, la morfogenesi e la differenziazione

1.6.1 NF-κB nella malattia celiaca.

Che ruolo ha il pathway di NF-κB nella malattia celiaca?

Il fattore di trascrizione NF-κB è un regolatore cruciale della risposta immunitaria adattativa e controlla l'attivazione, la proliferazione e la sopravvivenza dei linfociti. È ampiamente accettato che NF-κB sia un regolatore chiave dell'espressione genica inducibile nel sistema immunitario. Le risposte immunitarie sia innate che adattative, così come lo sviluppo e il mantenimento delle cellule e degli organi che compongono il sistema immunitario sono, in più fasi, sotto il controllo della famiglia di fattori di trascrizione NF-κB [45]. Inoltre, NF-κB è responsabile della trascrizione di geni che codificano un certo numero di citochine e

chemochine pro-infiammatorie [46]. È stato anche dimostrato che NF-κB è un importante mediatore di IL15, che è in grado di ridurre i livelli di claudina-2 nelle strutture epiteliali a giunzione stretta e porta ad un aumento della permeabilità para-cellulare [47], un fenomeno ricorrente, rilevante e persistente nella CD [48]. Inoltre, in uno studio recente, è stato dimostrato che gli effetti della gliadina sugli enterociti sono mediati dallo stress ossidativo, dall'attivazione di NF-κB e dalla sovra-regolazione di IL15 [49]. È stato anche dimostrato che la via di NF-κB è costitutivamente sovra-regolata nella mucosa CD [50]. In un lavoro condotto da Fernandez-Jimenez N. et. al., è stata studiata l'espressione di 93 geni correlati a NF-κB ed è stato confermato l'attivazione costitutiva di una parte considerevole dei "functional players" più centrali di questa via biologica nella CD. Sulla base delle caratteristiche e funzioni di NF-κB, unitamente al fatto che i polimorfismi genetici nei principali mediatori di NF-κB come REL e TNFAIP3 sono stati associati alla suscettibilità della CD [51], rendono questo "pathway" un candidato estremamente interessante per avere un ruolo causale di primo piano nello sviluppo della malattia. L'ipotesi di lavoro più accreditata al momento è che vi sia un'alterazione genetica sistemica nei pazienti celiaci che porta all'attivazione costitutiva e anomala della via NF-κB, segno distintivo della malattia [46].

2 Scopo

La ricerca di questo dottorato si è focalizzata sul ruolo del probiotico Lattobacillus Paracasei CBA L74, e nell'attivazione del complesso di mTOR e NF-κB in cellule epiteliali intestinali da parte della gliadina. I peptidi non digeriti della gliadina sono in grado di stimolare sia la risposta immune innata che quella adattativa e di causare il danno della mucosa intestinale tipico dell'intestino dei pazienti celiaci. Negli anni per i pazienti celiaci sono state proposte terapie alternative, in particolare una di queste si basa sul principio della distruzione dei peptidi della gliadina presenti nel cibo, mentre un altro metodo ha come obiettivo il blocco dell'entrata dei peptidi nell'epitelio intestinale, prevenendo l'attivazione della risposta

immune. I probiotici hanno caratteristiche che potrebbero essere utili in entrambe le aree di ricerca. In questo contesto e interessante notare che il Lactobacillo Paracasei CBA L74 (Trade Mark Plasmon) può prevenire l'entrata del peptide P31-43 nelle cellule epiteliali intestinali probabilmente interferendo con il traffico endocitico [52]. La proteina chinasi target della rapamicina (TOR) è un integratore chiave di fattori ambientali,

compresi nutrienti e fattori di crescita disponibili nello spazio extracellulare e anche un regolatore dello stress cellulare. In questo progetto abbiamo studiato gli effetti del peptide della gliadina non digerito P31-43 sul complesso di mTOR e la capacita del probiotico Lactobacillo Paracasei CBA L74 di prevenire gli effetti del P 31-43. Abbiamo fatto questo in cellule epiteliali intestinali; sia linee immortalizzate ($Caco_2$) che organoidi derivati da staminali intestinali. Inoltre, per capire cosa succedesse all'attività del probiotico dopo il processo digestivo abbiamo sottoposto il surnatante di Lactobacillus Paracasei CBA L74 al processo digestivo per mezzo di un sistema semi- automatizzato e ne abbiamo valutato gli effetti biologici.

L'obiettivo finale del progetto è incentrato sullo sviluppo di nuovi approcci nutrizionali e sul miglioramento della qualità di vita di pazienti affetti da malattia celiaca. Il programma di ricerca ha la potenzialità di sviluppare e tradurre in applicazioni cliniche nuovi approcci nutrizionali che possano portare a vantaggi per il Paese, ad aumento del benessere dei cittadini e a riduzione dell'onere finanziario per il Sistema Sanitario Nazionale.

3 Materiali e metodi

3.1 Modelli cellulari

In questo progetto abbiamo utilizzato diversi modelli cellulari:

- Le cellule $Caco_2$ sono una linea di cellule intestinali umane derivate da cellule di adenocarcinoma colon-rettale. Tali cellule sono immortalizzate, e rappresentano dunque una linea cellulare secondaria. Sebbene derivate da un carcinoma del colon, quando coltivate in condizioni specifiche le cellule si differenziano e si polarizzano in modo tale che il loro fenotipo, morfologicamente e funzionalmente, assomiglia agli enterociti che rivestono l'intestino tenue. Le cellule $Caco_2$ sono coltivate in un terreno di coltura Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, San Giuliano Milanese, Italia), arricchito con 10% di siero fetale bovino (FBS, GIBCO), 100 unità/ml di antibiotico penicillina/ streptomicina (GIBCO), e 1 mM di L-glutammina (GIBCO). Il mezzo è sostituito ogni due giorni. Le cellule sono coltivate in ambiente sterile, lavorando sotto cappa a flusso laminare verticale e tenute in incubatore a 37°C, in atmosfera umidificata, costituita dal 95% da aria e il 5% di CO2. La crescita cellulare è valutata tramite analisi al microscopio a contrasto di fase, per determinare il grado di confluenza delle piastre. Nonostante le cellule $Caco_2$ siano un modello cellulare utilizzato attualmente in diversi laboratori e, nonostante queste acquisiscono caratteristiche morfologiche e funzionali tipiche degli enterociti dell'intestino tenue, bisogna sottolineare un limite di tale modello, che non le rende idonee nel rappresentare il miglior modello intestinale in vitro. Il grande limite è che le cellule $Caco_2$ consistono in un solo tipo cellulare e falliscono nel riprodurre la diversità dei tipi di cellule presenti nell'epitelio intestinale, fallendo così nel riprodurre interamente la fisiologia dell'epitelio intestinale [53].

  Uno dei punti di innovazione di questo progetto è rappresentato dall'utilizzo degli organoidi intestinali. Tali organoidi forniscono un modello di intestino in vitro che può essere impiegato in maniera più generale non solo dalla industria alimentare. Gli organoidi sono definiti come aggregati di cellule che assumono una precisa conformazione tridimensionale, finendo con l'assomigliare a organi in miniatura. La capacità delle cellule che li compongono di organizzarsi e distribuirsi ordinatamente, li ha resi dei modelli cellulari in 3D

impareggiabili per conseguire informazioni nuove sullo sviluppo dei vari organi e sulle interazioni tra i tessuti che li formano. Gli organoidi sono in grado di replicare efficacemente l'organizzazione di un organo e la sua fisiologia quando sono stimolati con gli appropriati fattori di crescita esogeni ed in presenza di un analogo della membrana basale come il Matrigel. Gli organoidi si ottengono da cellule staminali; nel nostro caso da una a due biopsie duodenali per individuo sono state prese con ERGDS endoscopico standard durante la gastro duodenoscopia di routine e poste in tampone di isolamento da 10 ml ghiacciato (5,6 mmol/ L Na2HPO4 (Sigma S7907; Sigma-Aldrich, St Louis, MO), 8,0 mmol/L KH2PO4 (Sigma P5655; Sigma-Aldrich), 96,2 mmol/L NaCl (Sigma S5886; Sigma-Aldrich), 1,6 mmol/L KCl (Sigma P5405; Sigma-Aldrich), 43,4 mmol/L saccarosio (Fisher BP220-1; Thermo Fisher Scientific, Waltham, MA) e 54,9 mmol/L D-sorbitolo (Fisher BP439-500; Thermo Fisher Scientific) in acqua deionizzata. Le unità della cripta sono state isolate secondo il protocollo di Yuli Wang et al. [54] al quale abbiamo apportato piccole variazioni. Dopo 60 minuti i campioni di biopsia sono stati ulteriormente digeriti enzimaticamente con collagenasi (2 mg / ml, Sigma-Aldrich Milano, Italia) in tampone di lavaggio (WB): (penicillina (100 unità ml-1), streptomicina (0,1 mg ml-1), l-glutammina (2 mM) e FBS (10%, vol / vol) a DMEM / F12 con HEPES in ghiaccio per 30 minuti. Trascorsi i 30 min i campioni sono stati filtrati attraverso un filtro da 70μm (Falcon, Germania) e il colino risciacquato con ulteriori 10 ml di WB. Le unità della cripta sono state raccolte mediante centrifugazione a 500×g per 5 min. Il surnatante è scartato e le cripte vengono accuratamente risospese in Matrigel (Corning 35623 Milano, Italia) il quale permetterà la crescita tridimensionali degli organoidi. Una volta risospese viene piastrata una quantità di 40 μL in una well-plate da 48 pozzetti. La well-plate è messa in incubatore a 37 ° C, 5% di CO2 per 10 minuti per consentire al Matrigel di solidificarsi. Successivamente, 300 μl di terreno di coltura cellulare arricchito con opportuni stimoli (CM-S) è aggiunto a ciascun pozzo e sostituito ogni due giorni. La caratteristica struttura in 3D degli organoidi intestinali limita

la manipolazione sperimentale del modello in quanto nella forma 3D la parte apicale dell'epitelio quindi la parte dei villi si trova rivolta all'interno del Matrigel mentre la parte basale è rivolta verso le pareti della piastra. Questa struttura chiusa ha limitato la manipolazione sperimentale che come scopo ha quello di studiare l'effetto di sostanze che agiscono sul lato luminale dell'epitelio intestinale. Per superare questa problematica siamo passati da una coltura tridimensionale dell'organoide ad una coltura bidimensionale (2D). Nel passaggio della coltura da 3D a quella in 2D gli organoidi vengono piastrati aperti in una well-plate da 6 pozzetti pre-trattata con in Matrigel diluito 1:40 in tampone fosfato salino (PBS). Successivamente si rimuove il mezzo di coltura degli organoidi, si va poi ad eseguire un lavaggio con PBS addizionato con EDTA e si recupera tutto in una falcon. La falcon viene poi centrifugata a 800 rpm per 5 min alla temperatura di 4°C. Viene aspirato il PBS contenente EDTA e si aggiunge lo 0,25% di tripsina per 2 minuti. La tripsina viene neutralizzata aggiungendo il washing buffer e gli organoidi vengono dissociati spipettando più volte la soluzione.

La falcon viene nuovamente centrifugata a 800 rpm per 5 min a 4°C, il pellet risospeso nel mezzo di coltura e il tutto è piastrato nella well-plate precedentemente pretrattata.

Il terreno di coltura degli organoidi è preparato utilizzando due terreni di coltura differenti in rapporto 1:1. Advanced DMEM/F-12 [Invitrogen] 1 mM N-acetil-L-cisteina (Sigma, Germania), 1x N-2 integratori (Gibco, Germania), 1 × B-27®supplementi (Gibco, Germania), 50ng/ml fattore di crescita epidermico, 10mM nicotinamide (Sigma, Germania), 10nM Leu15-gastrina I (Sigma, Germania), 500 nM A8301 (inibitore per ALK4/5/7; Sigma, Germania), 10 μM SB202190 (inibitore della chinasi p38 MAP; Sigma, Germania) e 10 μM Y-27632 (inibitore rock p160; Tocris, Germania) in conformità con i protocolli di Sato et al. [55], VanDussen et al. [56] e Yuli Wang et al. [54] e il mezzo L-WRN. Quest ultimo è preparato a partire da cellule L di topo che esprimono Wnt3a, R-spondine noggin. Tali cellule sono state acquistate commercialmente (ATCC CRL-3276, Bio Tech Standards,

Germania) e il mezzo condizionato (L-WRN) è stato preparato secondo le istruzioni e il protocollo dell'azienda.

3.2 Western Blot

I lisati cellulari, sia derivanti dalle Caco$_2$ che dagli organoidi, sono analizzati tramite elettroforesi su gel di poliacrilammide in condizioni denaturanti (SDS-PAGE): si tratta di una tecnica immunochimica che permette di ottenere la separazione di una miscela eterogenea di proteine ottenuta dai lisati cellulari, sfruttando la differenza di peso molecolare esistente tra le diverse proteine. Il Sodio dodeci-solfato (SDS), un denaturante con carica negativa, è responsabile della denaturazione delle proteine, definisce un rapporto massa/carica pressoché costante, dato che, una molecola di SDS si lega ad una proteina ogni 2 residui amminoacidici. Questo significa che vengono soppresse le differenze di carica esistenti tra le diverse proteine, come pure le differenze rilevabili durante la corsa elettroforetica e imputabili ad eventuali strutture tridimensionali. Le proteine, separate esclusivamente sulla base del peso molecolare, vengono riconosciute mediante l'utilizzo di anticorpi specifici. Il gel di poliacrilammide è costituito da una fase superiore di Staking gel (1 ml di acrilamide/bis-acrilammide in rapporto 29:1 al 40%, 2,5 ml di Tris-HCL a ph 6.8, 100 µl di Ammonio persolfato APS, 50 µl di SDS 20%, 10 µl di TEMED) e da una fase inferiore di Separating gel (2,5 ml di acrilamide/bis-acrilammide in rapporto 29:1 al 40%, 2,5 ml di Tris-Hcl a ph 8,8, 50 µl di SDS 20%, 100 µl APS, 10µl di TEMED). Per la corsa elettroforetica è stato usato un buffer di corsa (14,4 g di Glicina 192 mM, 3,03 g Trizma 25 mM, 5 ml SDS 20% e 995ml acqua distillata). Le membrane sono successivamente trattate con una soluzione di blocking, con MILK al 5% (Milk Blotting BioRad 2,5 g, in 50 ml di TTBS 1X) per un'ora. Il TTBS è una soluzione utilizzata per il lavaggio delle membrane, ottenuta da acqua distillata (volume totale 500 ml), 20 ml di Tris-Hcl a ph 8.0 20 mM, 1 ml

di Tween 20 0,1% e 8,8 gr di NaCl. Successivamente le proteine sono riconosciute utilizzando diversi anticorpi, di seguito indicati:

- la proteina p4EBP è stata riconosciuta mediante anti-p4EBP di coniglio (CellSignaling Technology);
- la proteina LC3 è stata riconosciuta mediante anti-LC3 di coniglio (Cell-Signaling Technology);
- la proteina p62 è stata riconosciuta mediante anti-p62 di coniglio (CellSignaling Technology);
- la proteina pp70 è stata riconosciuta mediante anti-pp70 di coniglio (CellSignaling Technology);
- la proteina pY-NF-κB è stata riconosciuta mediante anti- pY-NF-κB di coniglio (CellSignaling Technology);
- la proteina p-mTor è stata riconosciuta mediante anti-p-mTor di coniglio (CellSignaling Technology);
- la proteina pY-ERK1/2 è stata riconosciuta mediante anti- pY-ERK1/2 di coniglio (CellSignaling Technology);
- La proteina GAPDH è stata riconosciuta mediante anti-GAPDH di topo (CellSignaling Technology).

Gli anticorpi primari sono utilizzati in un rapporto di 1:1000 in BSA al 5%, incubati per tutta la notte. A seguito delle procedure di lavaggio, il filtro viene incubato per 45 minuticon il relativo anticorpo secondario, in MILK al 3% con un rapporto di 1:3000; successivamente vengono ripetute le procedure di lavaggio con TTBS (3 volte per 5 minuti). Le bande sono visualizzate utilizzando una soluzione ECL (GE Healtcare, Amersham, Buckinghamshire, UK), con un’esposizione di circa 2 min. L'intensità della banda è stata valutata integrando tutti i pixel della banda dopo la sottrazione dello sfondo per calcolare la media dei pixel che circondano la banda.

3.3 Stimoli utilizzati sui modelli cellulari

Durante questi tre anni di dottorato sono stati eseguiti diversi esperimenti che coinvolgono l'utilizzo di vari stimoli che brevemente illustriamo nell'elenco sottostante.

- PTG: Prodotto peptico-triptico della gliadina; rappresenta tutta la gliadina digerita nella quale insieme ad altri peptidi è compreso anche il P 31-43.
- P31-43: è un peptide della gliadina che non viene digerito dal sistema digerente e che viene ritrovato intatto fino nelle feci umane; la sua sequenza è LGQQQPFPPQQPY.
- Post-biotico di Lactobacillus Paracasei CBA L74: mezzo di coltura batterica dal quale, in seguito alla crescita batterica, le cellule batteriche vengono eliminate.
- Peptide Heinz: peptide isolato dal post-biotico di LP, tramite frazionamento. Brevetto Heinz in corso di approvazione.

3.4 Condizioni di crescita batterica

Per la preparazione del surnatante del probiotico, abbiamo coltivato L. Paracasei CBA L74 in DMEM integrato con 10% di siero fetale di vitello (FCS, GIBCO), e 1-mM glutammina (GIBCO) fino a 109 CFU/ml come precedentemente descritto (Sarno et al., 2014). La coltura batterica è stata poi centrifugata a 3.000 rpm per 10 min e il surnatante è stato filtrato attraverso un filtro da 0 a 22 micron.

3.5 Immunofluorescenza

Le cellule vengono coltivate su vetrini sterili; questi vetrini vengono poi trasferiti in una piastra da 24 pozzetti e pretrattati e non con il post-biotico per 2 h e poi stimolate con PTG e P3-43 per 1h. Dopo la fissazione con paraformaldeide al 3% per 5 min a temperatura ambiente e la permeabilizzazione con Triton 0,2% (Biorad, Milano, Italia) per 5 min a temperatura ambiente, le cellule CaCo-2 sono state colorate per 1 h a temperatura ambiente con anticorpo anti-LC3II (Cell signalling Milano Italia). Gli anticorpi secondari Alexa-488 coniugati (Invitrogen) anti-rabbit per LC3II sono stati aggiunti ai copri-oggetti per 45 min a temperatura ambiente. I copri-oggetti, dopo il montaggio su vetrini, sono stati osservati al microscopio confocale (LSM 510 Zeiss, Milano, Italia), e le immagini sono state analizzate

con il software AIS Zeiss per valutare l'intensità di fluorescenza (FI) del campo microscopico in esame (Lania et al 2018). L'ingrandimento delle micrografie è stato lo stesso per tutte le figure riportate (obiettivo 63 ×).

3.6 Digestione Lactobacillus Paracaei CBA L74

Altro punto innovativo di questo progetto è quello di sottoporre il surnatante del probiotico Lactobacillus Paracasei CBA L74 al processo digestivo. Le analisi in vivo non possono sempre essere condotte a causa di motivi etici, vincoli tecnici o costi, ma una migliore comprensione del processo digestivo, specialmente nei bambini, potrebbe essere di grande aiuto nella prevenzione delle patologie legate all'alimentazione e nello sviluppo di nuove formule con benefici per la salute. In questo contesto, un sistema dinamico in vitro per simulare la digestione umana e, in particolare, la digestione infantile è stato sviluppato dalla Heinz per testare la biodisponibilità dei nutrienti dopo il processo di digestione. M-I.D.A. (Model of an Infant Digestive Apparatus) è un sistema di digestione semi-automatico dinamico in grado di simulare in vitro tutte le fasi della digestione dalla masticazione/ alla digestione duodenale. Per quanto altri sistemi di digestione in vitro esistano, M.I.D.A. è l'unico (allo stadio attuale delle conoscenze) che ricostruisce un apparato intestinale di un bambino di 6 mesi. La dinamica del sistema M.I.D.A è garantita dalla continuità del tratto gastrointestinale, che è composto da tre scomparti, che non sono considerati come semplici fiasche separate ma come distretti che interagiscono tra loro; inoltre con questo sistema di digestione è stato possibile prelevare e testare non solo il prodotto finale dell'intera digestione ma anche il prodotto delle digestioni dei singoli distretti. In ognuno di questi compartimenti abbiamo simulato la temperatura fisiologica, il pH e i profili enzimatici. Il surnatante del Probiotico Lactobacillus CBA L74 digerito tramite il sistema M.I.D.A. e il

risultato delle varie tappe di digestione sono state testate sul modello cellulare intestinale per valutarne gli effetti sulla via di mTOR.

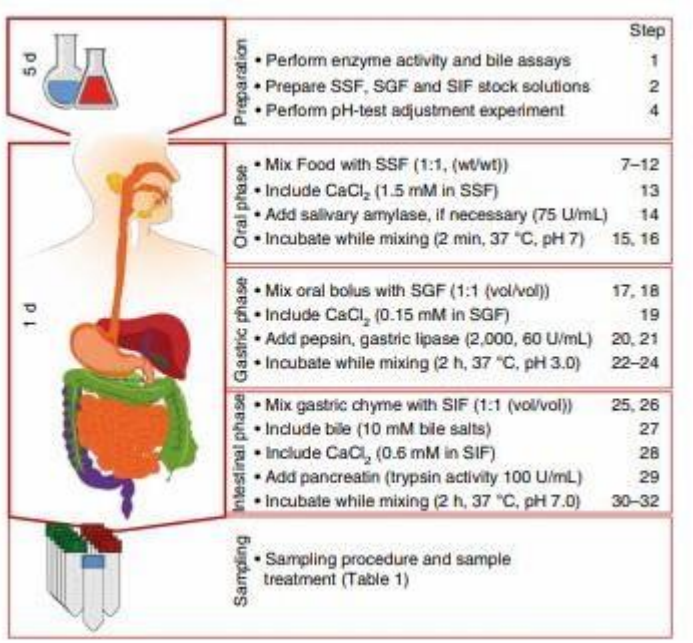


Fig.5 Diagramma di flusso del metodo di digestione INFOGEST 2.0

4 ANALISI STATISTICA E GRAFICI

L'analisi statistica e i grafici sono stati ottenuti usando il programma GraphPad Prism. Sono state calcolate la media e la deviazione standard dei risultati nei vari esperimenti; la significatività di questi è stata valutata mediante test T di Student accettando come significativi solo risultati che presentavano valori di $p<0,05$.

5 RISULTATI

5.1 I peptidi di gliadina non digeriti sono in grado di indurre la fosforilazione di mTOR

La proteina **mTOR** (acronimo di **mammalian target of rapamycin**, *bersaglio della rapamicina nei mammiferi*) è una chinasi a serina/treonina che integra una moltitudine di segnali extracellulari e stimoli intracellulari per guidare la crescita e la proliferazione cellulare.

Abbiamo studiato gli effetti del peptide di gliadina P31-43 sulle cellule $Caco_2$, una linea cellulare epiteliale intestinale che è sensibile alla gliadina, per ottenere una migliore comprensione dei meccanismi attraverso i quali questo peptide attiva il pathway di mTOR. Per determinare se il pathway di mTOR è attivo le cellule $Caco_2$ sono state trattate con PTGe P31-43 e successivamente tramite Western blotting abbiamo analizzato le forme fosforilate, quindi attive, di proteine che sono targets di mTOR, come p70S6 Kinase 1 (S6K1)e eIF4E Binding Protein (4EBP).

I livelli di espressione delle proteine p-mTOR, S6K1 e 4EBP sono aumentati rispetto alle cellule non trattate (NT). Quando le cellule $Caco_2$ vengono pretrattate con il post-biotico di Lactobacillus Paracasei CBA L74 per 1h e poi successivamente stimolate con peptidi di gliadina (PTG e P31-43), LP impedisce l'attivazione del pathway di mTOR Fig 6.

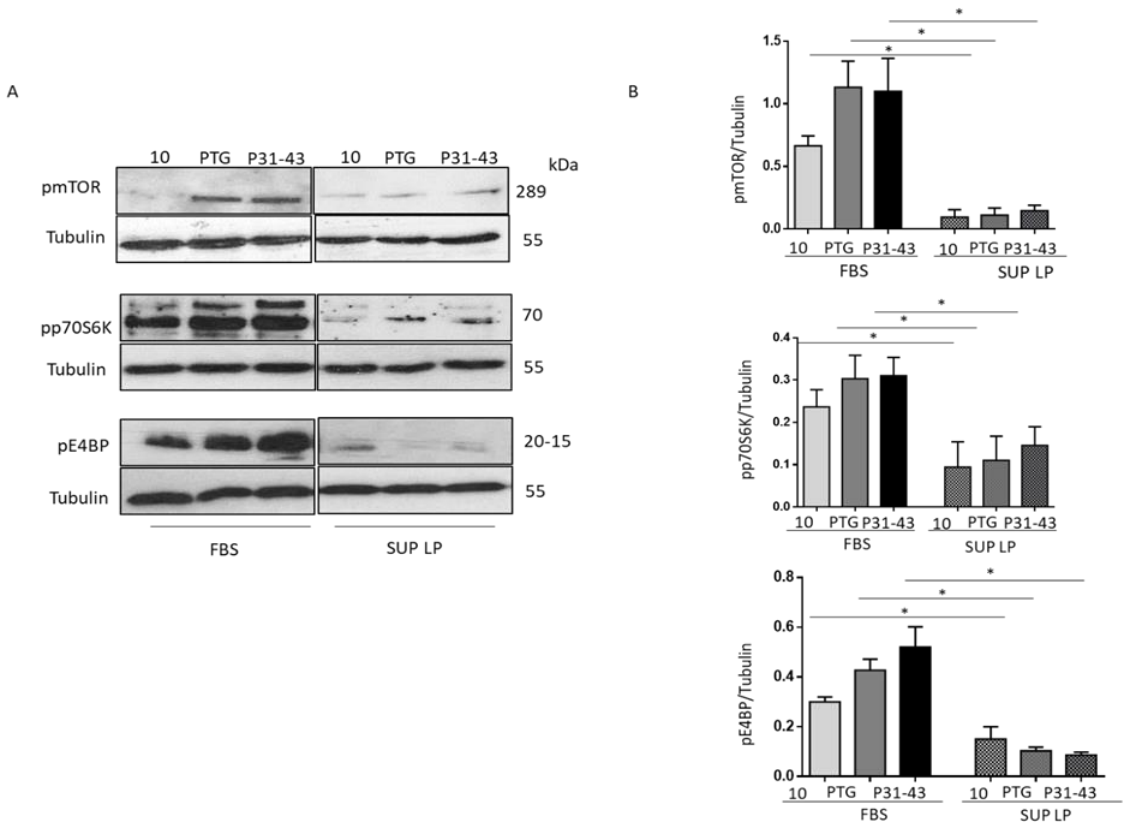


Figura 6. Il post-biotico di Lactobacillus Paracasei CBA L74 previene l'attivazione del pathway di mTor indotto dalla gliadina. I lisati proteici delle cellule $Caco_2$ non trattate (10%FBS) e delle cellule $Caco_2$ trattate con PTG e P31-43 per 30 minuti e pretrattate con LP per 1 ora sono stati analizzati mediante analisi Western blot usando anticorpi per p-mTOR, pp70s6ke p4EBP. La tubulina è stata usata come controllo del caricamento. I grafici sono rappresentativi di tre esperimenti indipendenti. Le colonne rappresentano la media, le barre la deviazione standard dell'intensità relativa di p-mTOR, pp70s6k e p4EBP rispetto alla proteina tubulina totale. Gli asterischi denotano differenze statisticamente significative (*$p<0.05$) tra le cellule pretrattate e non con il post-biotico di Lactobacillus Paracasei CBA L74.

5.2 I peptidi della gliadina riducono il pathway dell'autofagia

La proteina mTOR regola anche un altro pathway importante che è quello dell'autofagia; l'autofagiaè il più importante pathway cellulare deputato alla degradazione delle proteine e degli organelli citosolici "vecchi e/o mal funzionanti", ed è per questo che tale percorso biologico estende la durata della vita di molte cellule e quindi di molti organismi. La regolazione del processo autofagico può essere mediata dal pathway di mTOR, che agisce da segnale inibitorio. Poiché abbiamo osservato unaumento del pathway di mTOR dopo il trattamento con peptidi di gliadina, PTG e P31-43, abbiamovalutato se tali stimoli avessero influenza anche sul percorso autofagico.

Abbiamo analizzato LC3II, marker principale dell'autofagia, e la proteina p62, una proteina marker dell'attivazione dell'autofagia e quindi del flusso delle vescicole LC3II positive ai lisosomi per la degradazione. Dopo il trattamento con peptidi di gliadina, PTG e P31-43, mediante Western blotting in cellule Caco$_2$ abbiamo osservato una riduzione dei livelli di LC3II e un aumento di p62 confermando che la gliadina non solo è in grado di attivare il pathway di mTOR , ma anche di ridurrel'autofagia in cellula epiteliali intestinali (FIG.7A – B). Il pretrattamento con surnatante di LP per 3 ore previene gli effetti dei peptidi di gliadina sui livelli di LC3 e p62, riattivando sia la formazione di vescicole autofagiche che il flusso ai lisosomi (FIG.7A - B). Inoltre, questi dati sono stati confermati anche dall'immunofluorescenza. (FIG.7D).

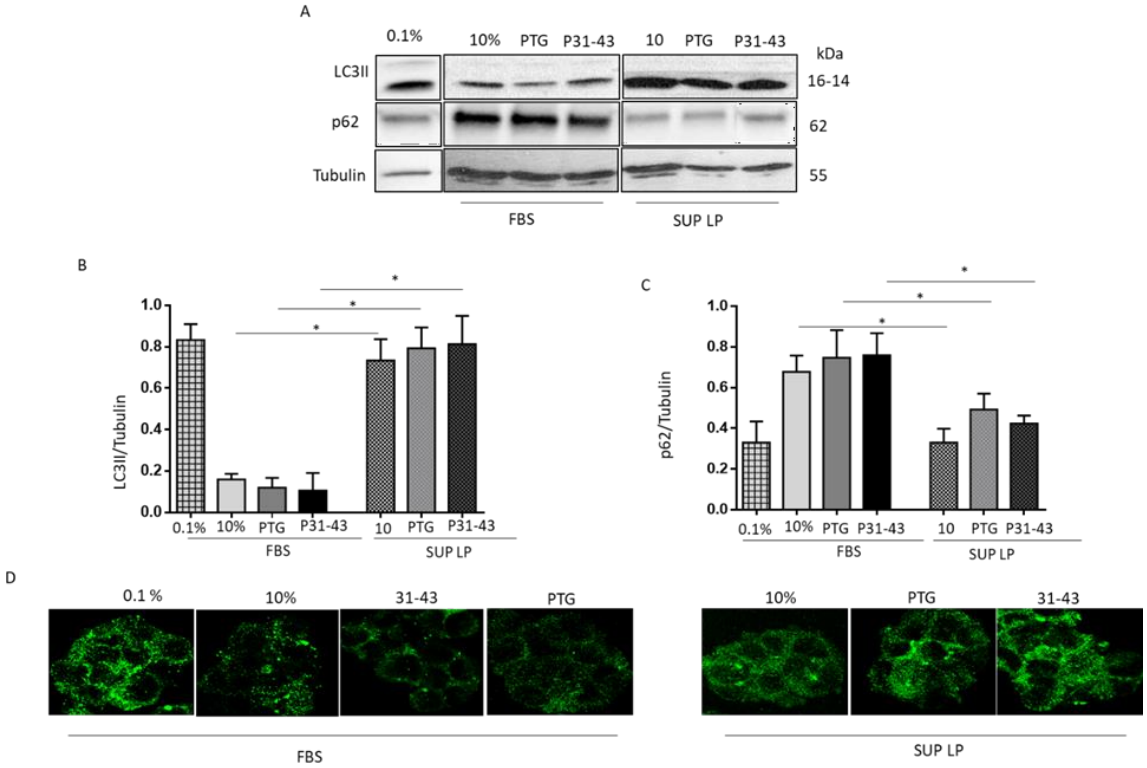


Figura 7. Il pretrattamento con il post-biotico di Lactobacillus Paracasei CBA L74 riduce gli effetti dei peptidi della gliadina.

Il post-biotico di Lactobacillus Paracasei CBA L74 riduce gli effetti dei peptidi della gliadina sul pathway dell'autofagia. I lisati proteici delle cellule Caco$_2$ non trattate (10%FBS) e delle cellule Caco$_2$ trattate con PTG e P31-43 per 1h e pretrattate con il post-biotico di LP per 2h sono state analizzate mediante analisi Western blot utilizzando anticorpi per LC3II e p62. La tubulina è stata usata come controllo di caricamento. Le immagini sono rappresentative di tre esperimenti indipendenti. Le colonne rappresentano la media, le barre la deviazione standard dell'intensità relativa di LC3II e p62 rispetto alla proteina tubulina totale. Gli asterischi denotano differenze statisticamente significative (*p<0.05) tra le cellule pretrattate e non con LP. La figura 2 D mostra l'immunofluorescenza di anti-LC3II in cellule Caco$_2$ non trattate (10%FBS) e cellule Caco$_2$ trattate con PTG e P31-43 per 1h e pretrattate con LP per 2h. Sono mostrate le immagini ottenute utilizzando un obiettivo 636 (zoom 26digitale).

PTG e P31-43 sono in grado di attivare sia NF-κB [57-58] che il pathway di mTOR, abbiamo valutato se il post biotico di LP fosse capace di prevenire gli effetti negativi dei peptidi della gliadina. A questo scopo le cellule $Caco_2$ sono state pretrattate con i peptidi della gliadina, PTG e P31-43 valutato tramite western blotting i livelli di espressione di pNF-κB. Come mostrato in figura 8A - B dopo trattamento sia con il PTG che con il peptide P31-43 i livelli pNF-κB sono aumentati in maniera statisticamente significativa, confermando un dato già presente in letteratura [57], ovvero che sia la gliadina che il peptide P31-43 hanno effetti infiammatori sulle cellule epiteliali. Successivamente le cellule $Caco_2$ sono state pretrattatecon il post-biotico di LP per 1 h e successivamente stimolate con peptidi della gliadina, PTG e P31-43; abbiamo poi nuovamente valutato tramite western blotting i livelli di espressione di pNF-κB. I dati hanno mostrato un aumento dei livelli di fosforilazione di pNF-κB dopo la stimolazione con PTG e P31-43, e la capacità del post biotico di LP di ridurre questa fosforilazione.

Poiché le MAPK sono coinvolte nell'attivazione di NF-κB dipendente da Iκ [59] abbiamo analizzato anche l'attivazione di ERK [57]. Dopo la stimolazione con PTG e P31-43, abbiamo confermato [57] un aumento della fosforilazione di ERK (FIG.8A – C). Il pre-trattamento con il post-biotico di LP era in grado di previene l'attivazione di ERK, dimostrando anche su questo read out che il post- biotico di LP può prevenire gli effetti della gliadina e del peptide P31-43. FIG.8A – C.

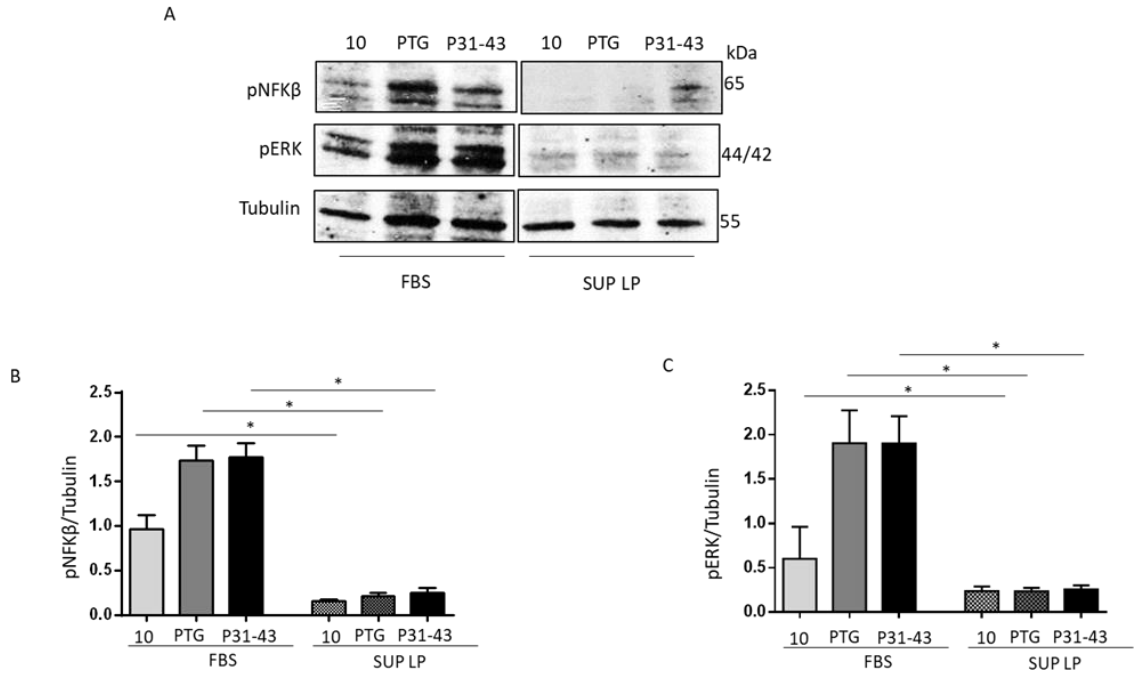


Figura 8. Il pretrattamento con LP ha diminuito la fosforilazione di NF-κB dopo il trattamento con P31-43 e PTG

I lisati proteici delle cellule $Caco_2$ non trattate (10%FBS) e delle cellule $Caco_2$ trattate con PTG e P31-43 per 30 minuti e pretrattate con il post-biotico di LP per 1 ora sono state analizzate mediante analisi Western blot utilizzando anticorpi per pNF-κB e pERK. La tubulina è stata usata come controllo del caricamento. Le immagini sono rappresentative di tre esperimenti indipendenti. Le colonne rappresentano la media, le barre la deviazione standard dell'intensità relativa di pNF-κB (B) e pERK (C) rispetto alla proteina totale della tubulina. Gli asterischi denotano differenze statisticamente significative (*$p<0.05$) tra le cellule trattate e non con LP.

5.3 La matrice riso, fermentata con Lactobacillus Paracasei CBA L74, attiva il pathway dell'autofagia.

Sempre più spesso i consumatori non consumano cibo solo per soddisfare la propria fame, ma si presta sempre più attenzione a prevenire tutte quelle malattie legate all'alimentazione e più in generale a migliorare la salute e la qualità della vita. In questo contesto negli ultimi anni si è evoluto sempre di più il concetto di cibo funzionale. Attualmente definiamo funzionale un alimento quando conferisce un beneficio alla salute umana. I probiotici fanno parte degli alimenti funzionali e sono ampiamente utilizzati per migliorare la salute dell'uomo. Di solito i probiotici vengono somministrati ai consumatori attraverso prodotti

quali yogurt, verdure o altri cibi fermentate [60]. Alla luce delle capacità biologiche attribuite al probiotico Lactobacillus Paracasei CBA L74, sia viste in questo lavoro, che in altri condotti precedentemente, quali proprietà anti-infiammatorie legate alla riduzione dell'ingresso del P31-43 nelle cellule $Caco_2$ [61], abbiamo valutato se con la fermentazione di diverse matrici alimentari quali, riso latte e fagioli, le proprietà biologiche del post-biotico derivante della fermentazione di tali matrici con Lactobacillus Paracasei CBA L74 potesse influenzare in positivo o negativo il pathway dell'autofagia. I risultati che abbiamo ottenuto ci mostrano che tra le tre matrici, riso, latte e fagioli, solo il riso fermentato con Lactobacillus Paracasei CBA L74 attiva il pathway dell'autofagia agendo sia sull'incremento delle vescicole LC3 positive che sul flusso autofagico con la riduzione di p62. FIG.9 A- B- C.

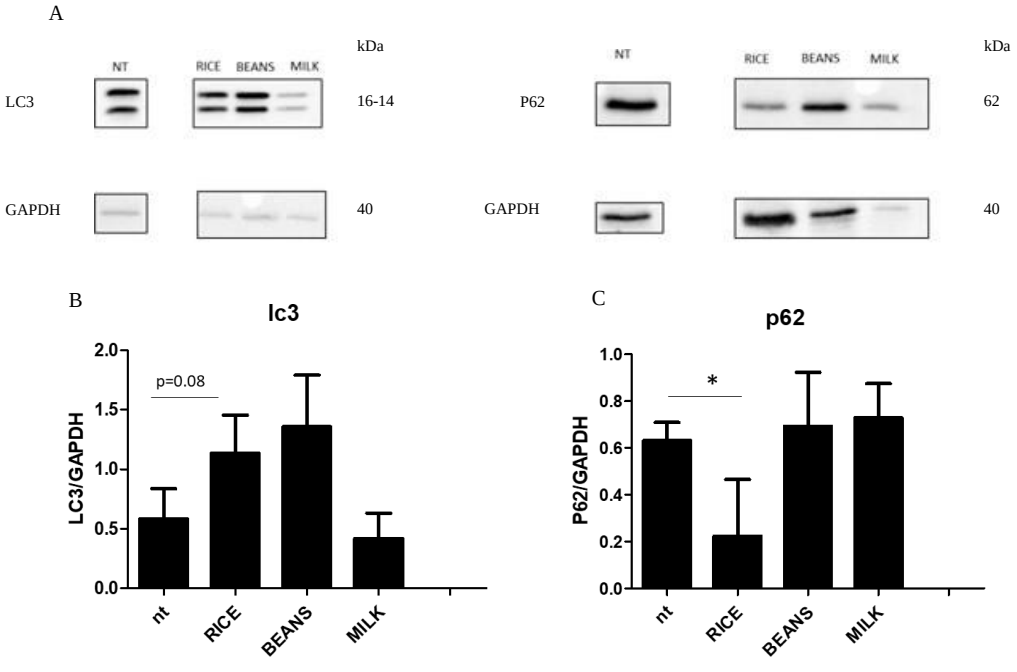


Figura 9. Il post-biotico derivante dalla fermentazione della matrice alimentare riso con LP attiva il pathway dell'autofagia.

I lisati proteici delle cellule $Caco_2$ non trattate (10%FBS) e delle cellule $Caco_2$ trattate per 3 ore con il post-biotico delle matrici riso latte e fagioli fermentate con LP sono state analizzate mediante analisi Western blot utilizzando anticorpi per LC3 e p62. GAPDH è stato usato come controllo del caricamento. Le immagini sono rappresentative di tre esperimenti indipendenti. Le colonne rappresentano la media, le barre la deviazione standard dell'intensità relativa di LC3 (B) e p62(C) rispetto alla proteina totale GAPDH. Gli asterischi denotano differenze statisticamente significative (*$p<0.05$) tra le cellule trattate e non con le matrici.

5.4 Un peptide isolato dal surnatante di Lactobacillus Paracasei CBA L74, denominato peptide Heinz, non mantiene tutte le capacità biologiche, del post-biotico completo, sul pathway dell'autofagia.

Cos'è che è presente nel post-biotico di Lactobacillus Paracasei che dona, a tale probiotico, questi effetti biologici?

Durante questo percorso di dottorato abbiamo cercato di capire se fosse possibile attribuire gli effetti biologici di tale probiotico ad una molecola presente nel post-biotico di Lactobacillus Paracasei; a tale scopo abbiamo testato sul nostro modello cellulare il peptide Heinz derivante dal post-biotico, tale peptide è stato isolato tramite frazionamento del post-biotico dalla Plasmon-Heinz che ha la sua sede operativa a Pozzuoli - Provincia: Napoli - Indirizzo: Via Campi Flegrei, 70 ex Comprensorio Olivetti Edificio N° 34. Questo peptide è stato testato sul nostro modello cellulare e i risultati che abbiamo ottenuto mostrano che questo peptide può attivare la prima parte del pathway dell'autofagia, portando un aumento di LC3 ma non è in grado di concludere tale pathway ed agire anche sul flusso autofagico con la riduzione di p62, anche se si presenta una tendenza alla riduzione Inoltre questo peptide non è in grado di ridurre il pathway di mTOR in quanto non ha alcun effetto sulla fosforilazione di E4BP. FIG.10 A- B- C- D

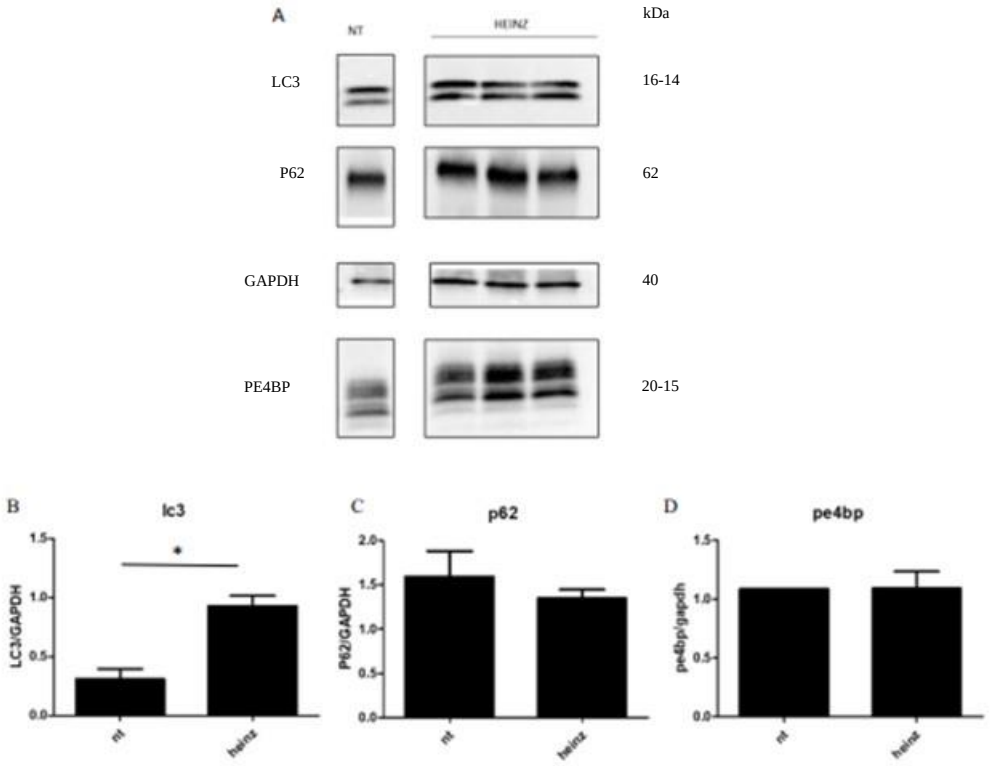


Figura 10. Il peptide Heinz isolato dal post-biotico di Lactobacillus Paracasei non mantiene tutti gli effetti biologici del post-biotico completo.

I lisati proteici delle cellule $Caco_2$ non trattate (10%FBS) e delle cellule $Caco_2$ trattate con il peptide Heinz per 3 ore sono state analizzate mediante analisi Western blot utilizzando anticorpi per LC3, p62 e pE4BP. GAPDH è stato usato come controllo del caricamento. Le immagini sono rappresentative di tre esperimenti indipendenti. Le colonne rappresentano la media, le barre la deviazione standard dell'intensità relativa di LC3 (B), p62(C) e pE4BP(D) rispetto alla proteina totale GAPDH. Gli asterischi denotano differenze statisticamente significative (*$p<0.05$) tra le cellule trattate e non con il peptide Heinz.

5.5 Il post-biotico di Lactobacillus Paracasei CBA L74 digerito attraverso il sisitema M.I.D.A. perde i suoi effetti biologici.

Tutto (o quasi) quello che viene a contatto con l'interno del nostro organismo passa per il sistema digerente e subisce tutti i processi che si svolgono nei suoi vari organi. Questi processi comprendono la digestione sia meccanica che chimica la quale avviene sia per variazioni di pH che si registrano lungo il sistema digerente sia per l'azione diretta di specifici enzimi sui diversi substrati che vengono a trovarsi nelle sedi digestive. Per valutare

quindi le future applicazioni, a carattere industriale, del Lactobacillus Paracasei CBA L74 è stato opportuno verificare se gli effetti biologici mostrati fino ad ora erano mantenuti anche in seguito al processo digestivo. Per poter procedere in questa direzione il post-biotico di Lactobacillus Paracasei CBA L74 è stato sottoposto ad un processo digestivo utilizzando il protocollo INFOGEST [62] attraverso il sistema M.I.D.A. (Model of an Infant Digestive Apparatus) messo a punto dal Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione, Università degli Studi di Napoli Federico II.

Dopo la procedura digestiva il post-biotico digerito è stato sottoposto ad un processo di dialisi in tampone TRIS HCl 1mM pH 7,4 utilizzando una membrana di cellulosa Spectral/Por dialysis membrane MWCO: 12-14 kD, prima di poter trattare le cellule con quest'ultimo. Tale procedura si è resa necessaria in quanto il modello cellulare non sopravviveva per più di 30 min nel momento in cui il post-biotico digerito veniva utilizzato tal quale. Dopo aver eseguito sia il processo digestivo che quello di dialisi abbiamo trattato il nostro modello cellulare con il post-biotico. I risultati mostrano che in seguito alla digestione il post biotico di Lactobacillus Paracasei perde i suoi effetti biologici.FIG.11 A-B-C-D.

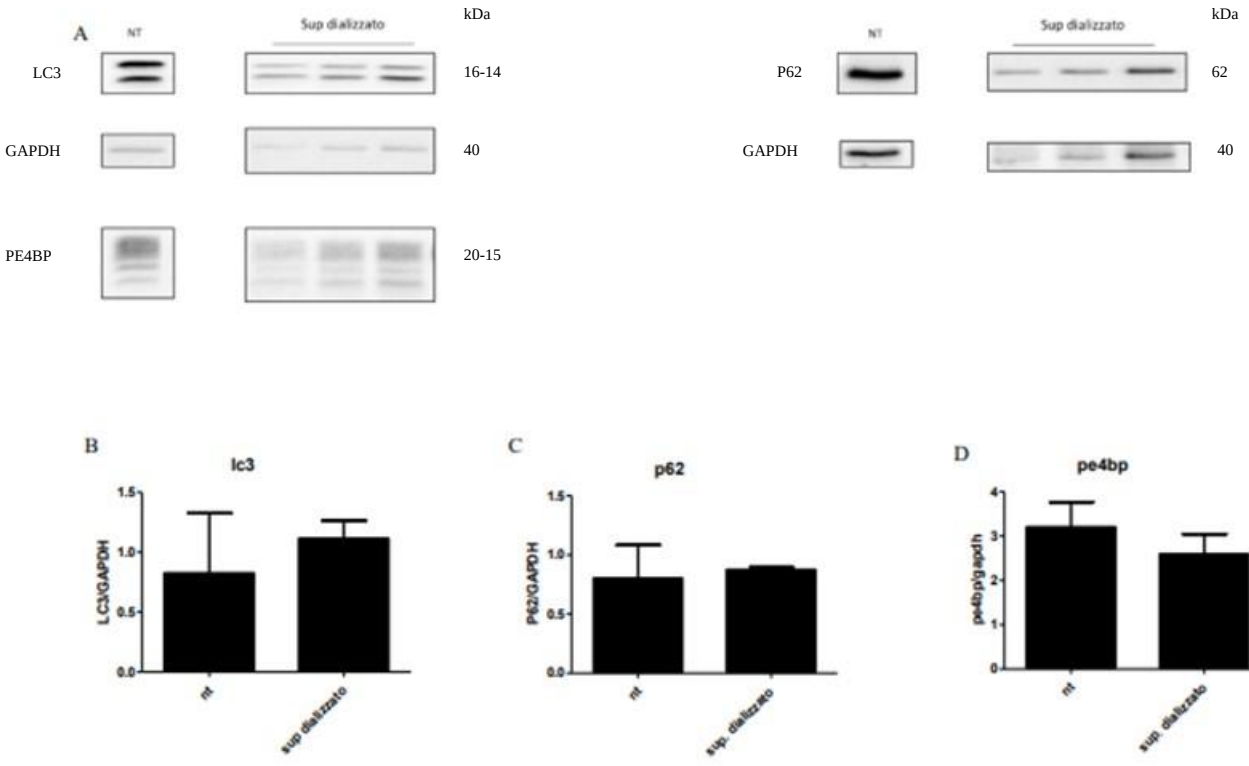


Figura 11. Il post-biotico di Lactobacillus Paracasei CBA L74 digerito attraverso il sisitema M.I.D.A. perde i suoi effetti biologici.

I lisati proteici delle cellule $Caco_2$ non trattate (10%FBS) e delle cellule $Caco_2$ trattate per 3 ore con il post-biotico digeritoe dializzato, sono state analizzate mediante analisi Western blot utilizzando anticorpi per LC3, p62 e pE4BP. GAPDH è stato usato come controllo del caricamento. Le immagini sono rappresentative di tre esperimenti indipendenti. Le colonne rappresentano la media, le barre la deviazione standard dell'intensità relativa di LC3 (B), p62(C) e pE4BP(D) rispetto alla proteina totale GAPDH.

5.6 Il post-biotico di Lactobacillus Paracasei CBA L74 riduce l'infiammazione negli organoidi di paziente.

Gli organoidi sono considerati un buon modello per studiare l'infiammazione e l'infezione dell'intestino [63]. Questi sono una versione miniaturizzata e semplificata di un organo prodotto in vitro in tre dimensioni che mostra una micro-anatomia realistica [64]. Gli organoidi intestinali derivano da cellule staminali delle cripte, per il corretto trattamento del lato apicale è necessario aprire gli organoidi in 2D, perché nella forma in 3D il lato apicale delle cellule si trova all'interno degli organoidi sferici. Negli organoidi derivanti da soggetti con malattia celiaca è stato trovato un aumento dei marcatori di infiammazione pNF-κB, pERK, IL1B e IL6 a livello di proteine e mRNA [64], e a differenza di altre malattie intestinali come le IBD, l'infiammazione negli organoidi CD era persistente, poiché i livelli di pNF-κB e pERK non diminuivano dopo più di dieci settimane in coltura senza alcun trattamento. Questo suggerisce che l'infiammazione negli organoidi CD non è residuale del tessuto di origine, ma è costitutiva. Sulla base di questo sfondo, degli effetti biologici mostrati dal post-biotico di Lactobacillus Paracasei e della capacità degli organoidi di rappresentare un modello cellulare più realistico e più vicino alla malattia abbiamo studiato gli effetti del post-biotico di Lactobacillus Paracasei CBA L74 utilizzando come modello cellulare gli organoidi. La prima cosa che abbiamo studiato è la differenza, nell'infiammazione, tra organoidi di soggetti controllo (CTR) ed organoidi di pazienti affetti da Malattia Celiaca (CD); paragonando questi due tipi di organoidi abbiamo trovato che in condizioni basali, cioè senza nessun trattamento, c'è una differenza statisticamente significativa nei livelli di espressione del marker d'infiammazione pNF-κB, il quale risulta maggiormente espresso negli organoidi di pazienti CD. FIG 12 A-B. Tale significatività viene persa nel momento in cui l'organoide di paziente CD viene trattato per 3 h con il post-biotico di Lactobacillus Paracasei CBA L74, indicando che tale trattamento porta ad una

riduzione nell'infiammazione negli organoidi CD FIG. 12 A-B. Inoltre, abbiamo osservato che negli organoidi di pazienti CD, il trattamento con il P31-43 porta ad un aumento statisticamente significativo dell'infiammazione e che il pretrattamento con il post-biotico riduce gli effetti infiammatori del P31-43. FIG.13 A-B.

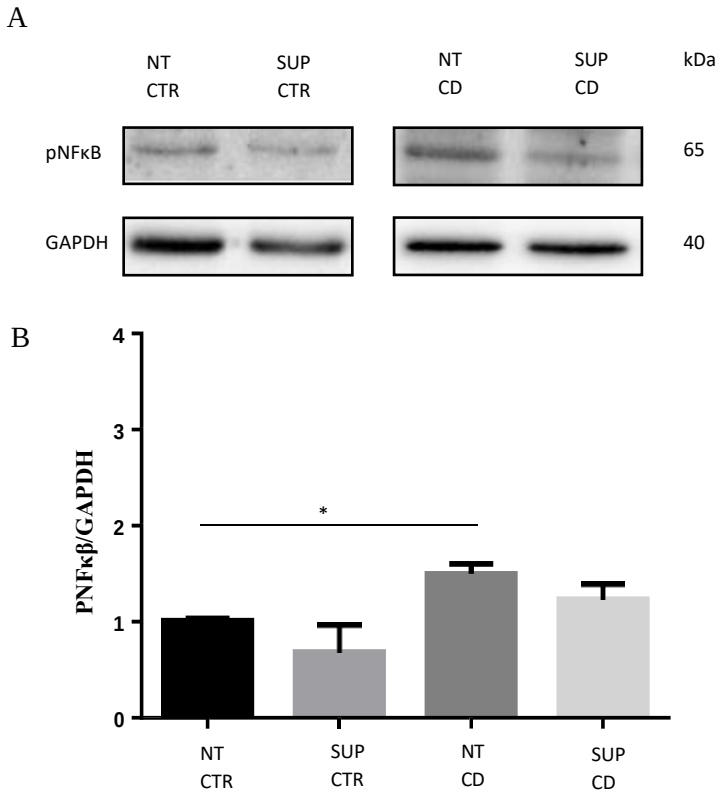


Figura 12. Il post-biotico di Lactobacillus Paracasei CBA L74 riduce l'infiammazione nell'organoide di paziente.

I lisati proteici degli organoidi di controllo (CTR) e soggetto paziente affetto da malattia celiaca (CD) non trattati e di organoidi di CTR e CD trattati per 3 ore con il post-biotico, sono stati analizzati mediante analisi Western blot utilizzando anticorpi per pNF-κB. GAPDH è stato usato come controllo del caricamento. Le immagini sono rappresentative di tre esperimenti indipendenti. Le colonne rappresentano la media, le barre la deviazione standard dell'intensità relativa di pNF-κB rispetto alla proteina totale GAPDH.

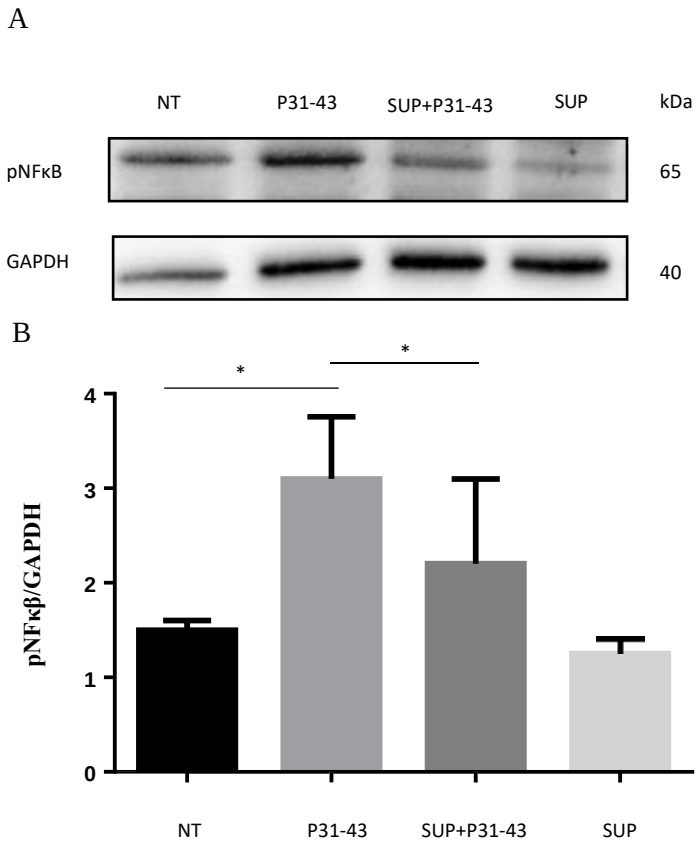


Figura 13. Il post-biotico di Lactobacillus Paracasei CBA L74 previene gli effetti del p31-43 nell'organoide di paziente.

I lisati proteici degli organoidi di paziente (CD) non trattati e trattati con P31-43 per 30 minuti e pretrattati con il post-biotico di LP per 3 ore, sono stati analizzati mediante analisi Western blot utilizzando anticorpi per pNF-κB. GAPDH è stato usato come controllo del caricamento. Le immagini sono rappresentative di tre esperimenti indipendenti. Le colonne rappresentano la media, le barre la deviazione standard dell'intensità relativa di pNF-κB rispetto alla proteina totale GAPDH.

5.7 Analisi degli organoidi attraverso la microscopia elettronica (EM)

Nel corso degli anni, l'approccio ultrastrutturale allo studio delle funzioni cellulari ha dato prova della sua validità sia nella scoperta che nella conferma di processi e meccanismi cellulari. Due esempi sono rappresentati da apoptosi e fagocitosi, inclusa l'autofagocitosi o autofagia. Nel primo caso, la definizione di un nuovo processo di morte cellulare è stata

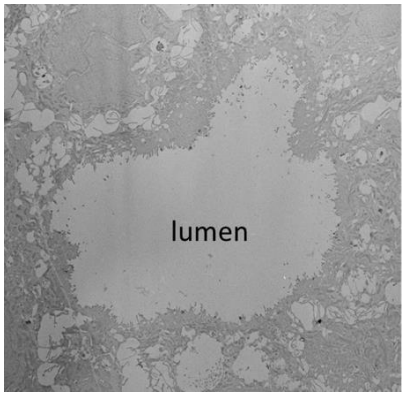
fornita dopo aver visto le caratteristiche peculiari di una cellula apoptotica e la sua strana distribuzione della cromatina. Per quanto riguarda il processo fagocitotico, inclusa l'autofagocitosi, la microscopia elettronica (EM) è ancora inestimabile nella dimostrazione della comparsa di questo meccanismo. In generale, l'analisi EM è considerata come una rappresentazione morfologica del tessuto, dando così la possibilità di osservare la presenza delle principali specie chimiche (proteine, acidi nucleici, carboidrati, lipidi) che costituiscono una cellula o un tessuto. Inoltre, la microscopia elettronica permette un'analisi morfologica molto fine del campione, permettendo di analizzare anche altri processi cellulari strettamente connessi con la funzione epiteliale, come le interazioni cellula-cellula, o cellule substrato. Nell'ultima fase del percorso di dottorato abbiamo inviato presso il laboratorio del Professore Zimmer a Giessen (Germania), esperto della Malattia Celiaca e di elettro-microscopia della mucosa intestinale, i campioni di organoidi di CD e CTR fissati per essere analizzati tramite microscopia elettronica. L'analisi ancora parziale di questi campioni è stata principalmente volta a verificare due fenotipi principali:

1) valutare la struttura degli organoidi dopo la procedura di fissazione

2) confrontare gli organoidi di soggetto CD con mucosa duodenale normale dal punto di vista ultrastrutturale.

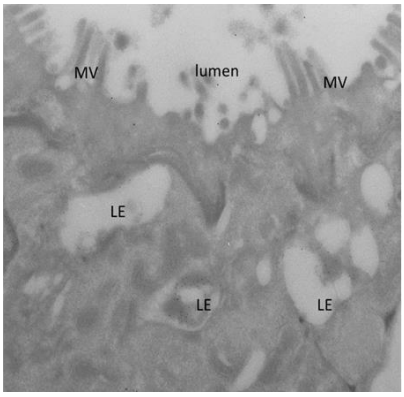
Dall'analisi di questi campioni sono emerse alcune caratteristiche interessanti; la prima da sottolineare è che la struttura circolare degli organoidi è preservata durante l'intera procedura della fissazione, indicando che questa non altera la struttura dell'organoide. Inoltre, è stato possibile valutare alcune caratteristiche basali degli enterociti come microvilli, giunzioni strette, vacuoli, mitocondri e nucleo. In particolare, è stata notata negli organoidi CD una elevata numerosità degli endosomi tardivi e una alterazione dei microvilli che appaiono piuttosto tozzi, con numero e densità ridotto rispetto agli enterociti di biopsie duodenali di soggetti controllo. Nell'insieme questi dati confermano che negli enterociti di soggetti CD ci sono alterazioni strutturali compatibili con un difetto di autofagia. FIG. 14.

Fig.14 Organoidi di paziente CD, allo stato basale, al microscopio elettronico.



CD patient 217, organoid overview

magnification x 1.540



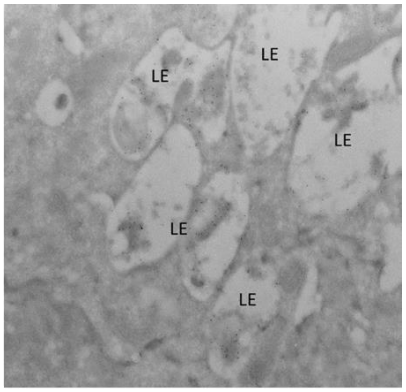
CD patient 217

Aminopeptidase 12nm gold particles

Cathepsin D 6 nm GP gold partices

magnification x 13.800

LE=late endosome, MV, microvilli



CD patient 217, late endosomes

Aminopeptidase 12nm gold particles

Cathepsin D 6 nm GP gold partices

magnification x 17.700

6 Discussione

In questo progetto, abbiamo studiato gli effetti della gliadina sul pathway di mTOR/autofagia e sull'infiammazione nelle cellule epiteliali e abbiamo anche valutato le capacità biologiche del post-biotico di Lactobacillus Paracasei (LP) in due modelli cellulari differenti, le cellule $Caco_2$ e gli organoidi. mTOR è una tirosin-chinasi che è in grado di regolare la proliferazione cellulare e inibire la via dell'autofagia. È regolata da segnali, come fattori di crescita e nutrienti, che guidano la crescita e la proliferazione cellulare. La via dell'autofagia si innesca quando mTOR è defosforilato, tale processo biologico permette le cellule e quindi

gli organismi di mantenersi in salute più a lungo, è una via strettamente connessa con la rigenerazione cellulare, consiste in un processo di digestione cellulare, che porta alla rimozione di macromolecole e organelli danneggiati al fine di mantenere l'omeostasi cellulare [35]. Le cellule $Caco_2$, un modello di cellule epiteliali intestinali, sono state utilizzate per studiare gli effetti della gliadina sulla via di mTOR e dell'autofagia. Sia il peptide P31-43 che il prodotto peptico-triptico della gliadina (PTG) sono in grado di attivare mTOR e il pathway a valle attraverso l'attivazione delle proteine E4BP e 70S6K. Inoltre, sia P31-43 che PTG sono in grado di attivare NF-kB nelle cellule $Caco_2$. Poiché l'attivazione di mTOR induce una riduzione dell'autofagia, abbiamo studiato l'attività di P31-43 e PTG sull'autofagia stessa, dimostrando una riduzione delle vescicole LC3-positive. Inoltre, in presenza di P31-43 e PTG, il flusso autofagico verso i lisosomi è diminuito come è dimostrato dall'aumento del marker p62. Presi insieme, questi dati dimostrano che p31-43 e PTG inducono una riduzione sia delle vescicole autofagiche che del loro flusso verso i lisosomi.

Studi recenti hanno indagato la capacità dei probiotici di prevenire gli effetti della gliadina in vivo. I loro effetti sono stati studiati sia in soggetti con malattia celiaca (CD) che in soggetti potenziali [65]. Nei soggetti con CD, c'è un parziale miglioramento dei sintomi gastrointestinali, se presenti, e una modifica del microbiota intestinale caratterizzato da un aumento dei Bifidobatteri, mentre nei potenziali, i pro-biotici non hanno mostrato alcun effetto nel prevenire la progressione della malattia. Presi insieme, questi dati indicano che l'uso di probiotici può agire sui sintomi gastrointestinali della malattia celiaca, procurando un effetto migliorativo, ma hanno poco effetto sul corso della malattia. Più recentemente, l'interesse del mondo scientifico si è concentrato sui post biotici Un post biotico è definito come una "preparazione di microrganismi inanimati e/o dei loro componenti: fattori solubili secreti da batteri vivi o rilasciati dopo la loro lisi, compresi vari componenti della superficie cellulare, acido lattico, acidi grassi a catena corta (SCFA) e peptidi bioattivi che conferiscono

un beneficio alla salute dell'ospite". A tutt'oggi non ci sono reports sugli effetti dei post-biotici nella malattia celiaca. In questo studio, abbiamo investigato la capacità del post-biotico del LP (Lactobacillus Paracasei CBA L74) di prevenire gli effetti della gliadina sulle cellule $Caco_2$ in vitro. Abbiamo usato cellule $Caco_2$ per studiare gli effetti della gliadina dopo il pretrattamento con il post-biotico di LP sulle vie mTOR e autofagia e NF-κB. Abbiamo dimostrato che, il pretrattamento con il post-biotico di LP è in grado di inibire l'attività di mTOR sia quando le cellule sono stimolate con il peptide P31-43 che con il PTG. Di conseguenza, anche NF-κB è risultato ridotto.

Inoltre, abbiamo studiato su cellule $Caco_2$ l'attività di P31-43 , di PTG e del pretrattamento con il post-biotico di LP sull'autofagia, dimostrando che i peptidi della gliadina , P31-43 e PTG, portano ad una riduzione del pathway autofagico mentre il pretrattamento con il post-biotico di LP inibisce gli effetti dei peptidi della gliadina portando un aumento delle vescicole autofagiche evidenziato dall'aumento del marcatore LC3 e ad un aumento del flusso autofagico verso i lisosomi, evidenziato dalla riduzione del marker p62. Abbiamo poi valutato se con la fermentazione di matrici alimentari, in particolare di riso, latte e fagioli, le proprietà biologiche del post-biotico derivante della fermentazione di tali matrici con Lactobacillus Paracasei CBA L74 potesse influenzare in positivo o negativo il pathway dell'autofagia. I risultati ci hanno mostrato che tra le tre matrici solo il riso fermentato con Lactobacillus Paracasei CBA L74 attiva il pathway dell'autofagia agendo sia su LC3 che su p62, mentre gli altri fermenti o sono solo parzialmente attivi (fagioli) o completamente inattivi (latte). Abbiamo inoltre cercato di evidenziare una molecola specifica, presente nel post-biotico, verso la quale potessero essere ricondotti gli effetti biologici che il post-biotico di LP ha dimostrato di avere, e a tal proposito abbiamo testato sul modello cellulare delle $Caco_2$ il peptide Heinz isolato dalla Plasmon-Heinz, questo peptide è capace di attivare la prima parte del pathway dell'autofagia, portando un aumento di LC3 ma non è in grado di

concludere tale pathway ed agire anche sulla riduzione di p62, ne tantomeno è capace di ridurre il pathway di mTOR.

Dato che uno degli obiettivi finali del progetto è quello di sviluppare di nuovi approcci per migliorare la qualità di vita dei soggetti con malattia celiaca è stato opportuno verificare se gli effetti biologici mostrati fino ad ora erano mantenuti anche in seguito al processo digestivo. Queste prove hanno dimostrato che in seguito alla digestione il post-biotico di Lactobacillus Paracasei CBA L74 perde i suoi effetti biologici; ciò suggerisce che il processo digestivo altera gli effetti del post-biotico.

Recentemente, Freire R et [66] al utilizzando organoidi intestinali sviluppati da biopsie duodenali di pazienti non celiaci (CTR) e celiaci (CD) hanno analizzato il ruolo delle molecole derivate dal microbiota nel modulare la risposta dell'epitelio al glutine. Hanno selezionato tre bioprodotti batterici: butirrato, lattato e PSA derivati da Bacteroides fragilis Tutti i bioprodotti hanno esercitato un effetto protettivo globale riducendo la secrezione di citochine pro-infiammatorie innescate dal PTG [67]. Utilizzando gli organoidi intestinali derivati da pazienti celiaci (CD) abbiamo analizzato l'effetto della gliadina dopo pretrattamento post-biotico di LP sul marcatore di infiammazione NF-κB dimostrando una riduzione. Inoltre, grazie all’uso della microscopia elettronica siamo riusciti ad analizzare organoidi di paziente in condizioni basali e notare la presenza di microvilli i quali appaiono piuttosto tozzi, con numero e densità ridotto rispetto alle biopsie duodenali. L'uso di organoidi in vitro derivati da pazienti per studiare la patogenesi della CD potrebbe essere un nuovo strumento per studiare ulteriormente il trattamento e la prevenzione della CD. In conclusione, il post-biotico di LP è in grado di prevenire in vitro gli effetti dei peptidi di gliadina sul pathway di mTOR, autofagia e NF-κB. Questi studi pre-clinici sono una buona base per attivare studi clinici nei pazienti celiaci per prevenire gli effetti pro-infiammatori dei peptidi di gliadina. In particolare, sarebbe interessante testare l'effetto del post-biotico, opportunamente protetto per resistere alla fase digestiva, per la sua capacità di prevenire la

malattia in soggetti potenziali, che hanno anticorpi anti-tranglutaminasi, ma non hanno ancora sviluppato la lesione intestinale tipica della malattia celiaca.

Attualmente, l'unico trattamento disponibile per un paziente con malattia celiaca è una rigorosa dieta senza glutine, Nonostante i migliori sforzi dei pazienti, alcuni soggetti possono risultare in continua esposizione a causa di contaminazione incrociata o tracce di glutine nel cibo. Questi rischi potrebbero, in alcuni casi, compromettere la salute e la qualità della vita di questi soggetti. È quindi generalmente utile studiare i composti che possono prevenire gli effetti infiammatori della gliadina con la speranza di ridurre il peso di vivere con la malattia celiaca e migliorare i risultati di salute a lungo termine.

Bibliografia

1. Veldhoen M., Brucklacher-Waldert V. Dietary influences on intestinal immunity. Nat. Rev. Immunol. 2012;12:696–708. [PubMed] [Google Scholar]

2. Marsh M.N. Clinical and pathological spectrum of coeliac disease. Gut. 1993;34:1740–1741. [PMC free article] [PubMed] [Google Scholar]

3. Marsh M.N., Loft D.E., Garner V.G. Time/dose responses of coeliac mucosae to graded oral challenges with Frazer's fraction III of gliadin. Eur. J. Gastroenterol. Hepatol. 1992;4:667–674. [Google Scholar]

4. Shan L., Molberg Ø., Parrot I., Hausch F., Filiz F., Gray G.M., Sollid L.M., Khosla C. Structural basis for gluten intolerance in Celiac Sprue. Science. 2002;297:2275–2279. [PubMed] [Google Scholar]

5. Nanayakkara M., Lania G., Maglio M., Discepolo V., Sarno M., Gaito A., Troncone R., Auricchio S.Auricchio R., Barone M.V. An undigested gliadin peptide activates innate immunity and proliferative signaling in enterocytes: the role in celiac disease. Am. J. Clin. Nutr. 2013;98:1123–1135. [PubMed] [Google Scholar]

6. Maria Vittoria Barone, Riccardo Troncone, Salvatore Auricchio. Gliadin Peptides as Triggers of the Proliferative and Stress/Innate Immune Response of the Celiac Small Intestinal Mucosa. Int J Mol Sci. 2014 Nov; 15(11): 20518–20537.

7. Marsh M.N., Crowe P.T. Morphology of the mucosal lesion in gluten sensitivity. Baillieres Clin. Gastroenterol. 1995;9:273–293. [PubMed] [Google Scholar]

8. Nanayakkara M., Lania G., Maglio M., Discepolo V., Sarno M., Gaito A., Troncone R., Auricchio S., Auricchio R., Barone M.V. An undigested gliadin peptide activates innate immunity and proliferative signaling in enterocytes: the role in celiac disease. Am. J. Clin. Nutr. 2013;98:1123–1135. [PubMed] [Google Scholar]

9. Molberg, O.; Mcadam, S.N.; Körner, R.; Quarsten, H.; Kristiansen, C.; Madsen, L.; Fugger, L.; Scott, H.; Norén, O.; Roepstorff, P.; et al. Tissue transglutaminase selectively modifies gliadin

peptides that are recognized by gut-derived T cells in celiac disease. Nat. Med. 1998, 4, 713–717. [CrossRef] [PubMed]

10. FAO/WHO Expert Consultation. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria (2001). www.fao.org

11. Ministero della Salute. Linee Guida Nutrizione/Probiotici.

12. Gibson, G.R.; Roberfroid, M.B. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. J. Nutr. 1995, 125, 1401–1412. [CrossRef]

13. Belkaid, Y.; Hand, T.W. Role of the microbiota in immunity and inflammation. Cell 2014, 157, 121–141. [CrossRef] [PubMed]

14. Di Gioia, D.; Aloisio, I.; Mazzola, G.; Biavati, B. Bifidobacteria: Their impact on gut microbiota composition and their applications as probiotics in infants. Appl. Microbiol. Biotechnol. 2014, 98, 563–577. [CrossRef]

15. Losurdo, G.; Principi, M.; Iannone, A.; Ierardi, E.; Di Leo, A. The interaction between celiac disease and intestinal microbiota. J. Clin. Gastroenterol. 2016, 50, S145–S147. [CrossRef]

16. Olivares, M.; Benítez-Páez, A.; de Palma, G.; Capilla, A.; Nova, E.; Castillejo, G.; Varea, V.; Marcos, A.; Garotte, J.A.; Polaco,I.; et al. Increased prevalence of pathogenic bacteria in the gut microbiota of infants at risk of developing celiac disease: The PROFICEL study. Gut Microbes 2018, 9, 551–558. [CrossRef]

17. Nistal, E.; Caminero, A.; Vivas, S.; Ruiz de Morales, J.M.; Sáenz de Miera, L.E.; Rodriguez-Aparicio, L.B.; Casqueiro, J. Differences in faecal bacteria populations and faecal bacteria metabolism in healthy adults and celiac disease patients. Biochimie 2012, 94, 1724–1729. [CrossRef] [PubMed]

18. De Palma, G.; Nadal, I.; Collado, M.C.; Sanz, Y. Effects of a gluten-free diet on gut microbiota and immune function in healthyadult human subjects. Br. J. Nutr. 2009, 102, 1154–1160. [CrossRef] [PubMed]

19. Gianfrani C, Auricchio S. La malattia celiaca: la ricerca oggi e le prospettive future. Proped, 2008;38:18-26

20. Barone MV, Gimigliano A, Castoria G, et al. Growth factor-like activity of gliadin, an alimentary protein: implications for coeliac disease. Gut 2007;56:480-8

21. Barone MV, Zanzi D, Maglio M, et al. Gliadin-mediated proliferation and innate immune activation in celiac disease are due to alterations in vesicular trafficking. PLoS One 2011;6:e17039

22. Nanayakkara M, Lania G, Maglio M, et al. An undigested gliadin peptide activates innate immunity and proliferative signaling in enterocytes: the role in celiac disease. Am J Clin Nutr 2013;98:1123-35, b.

23. Soares FL, de Oliveira Matoso R, Teixeira LG, et al. Gluten-free diet reduces adiposity, inflammation and insulin resistance associated with the induction of PPAR-alpha and PPAR-gamma expression. J Nutr Biochem 2013;24:1105-11.

24. Junker Y, Zeissig S, Kim SJ, et al. Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4. J Exp Med 2012;209:2395- 408

25. Savilahti E, Ormälä T, Saukkonen T. Jejuna of patients with insulin-dependent diabetes mellitus (IDDM) show signs of immune activation. Clin Exp Immunol 1999;116:70-7.

26. Westerholm-Ormio M, Vaarala O, Pihkala P, et al. Immunologic activity in the small intestinal mucosa of pediatric patients with type 1 diabetes. Diabetes 2003;52:2287-95.

27. Lefebvre DE, Powell KL, Strom A, et al. Dietary proteins as environmental modifiers of type 1 diabetes mellitus. Annu Rev Nutr 2006;26:175-202.

28. Troncone R, Franzese A, Mazzarella G, et al. Gluten sensitivity in a subset of children with insulin dependent diabetes mellitus. Am J Gastroenterol 2003;98:590-5.

29. Auricchio R, Paparo F, Maglio M, et al. In vitro-deranged intestinal immune response to gliadin in type 1 diabetes. Diabetes 2004;53:1680-3.

30. Shepherd SJ, Lomer MC, Gibson PR. Short Chain Carbohydrates and functional gastrointestinal disorders. Am J Gastroenterol 2013;108:707-17.

31. Associazione Italiana Celiachia. “Giovani Monteleone , progetto triennale – area celiaca- settore immunologia”

32. Beugnet A, Wang X, Proud CG. Target of rapamycin (TOR)-signaling and RAIP motifs play distinct roles in the mammalian TOR-dependent phosphorylation of initiation factor 4E-binding protein 1. 2003 Oct 17;278(42):40 717-22. Epub 2003 Aug 11

33. Sarkar, S. (2013) Regulation of autophagy by mTOR-dependent and mTORindependent pathways: autophagy dysfunction in neurodegenerative diseases and therapeutic application of autophagy enhancers. Biochem. Soc. Trans. 41, 1103–1130.

34. Dan HC1, Cooper MJ, Cogswell PC, Duncan JA, Ting JP, Baldwin AS. Aktdependent regulation of NF-{kappa}B is controlled by mTOR and Raptor in association with IKK. 2008 Jun 1;22(11):1490-500.

35. Tan CC, Yu JT, Tan MS, Jiang T, Zhu XC, Tan L. Autophagy in aging and neurodegenerative diseases: implications for pathogenesis and therapy. Neurobiol Aging. 2013, 35: 941-957.

36. Oeckinghaus A, Ghosh S . The NF-kappaB family of transcription factors and its regulation. Cold Spring Harb Perspect Biol 2009; 1: a000034.

37. Sun SC, Chang JH, Jin J . Regulation of nuclear factor-kappaB in autoimmunity. Trends Immunol 2013; 34: 282–289.

38. Sun SC . Non-canonical NF-kappaB signaling pathway. Cell Res 2011; 21: 71–85.

39. Vallabhapurapu S, Karin M . Regulation and function of NF-kappaB transcription factors in the immune system. Annu Rev Immunol 2009; 27: 693–733.

40. Karin M, Delhase M . The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. Semin Immunol 2000; 12: 85–98.

41. Sun SC, Ley SC . New insights into NF-kappaB regulation and function. Trends Immunol 2008; 29: 469-478.

42. Israel A . The IKK complex, a central regulator of NF-kappaB activation. Cold Spring Harb Perspect Biol 2010; 2: a000158.

43. Beinke S, Ley SC . Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. Biochem J 2004; 382: 393–409.

44. Hayden MS, Ghosh S . Shared principles in NF-kappaB signaling. Cell 2008; 132: 344–362.

45. Nora Fernandez-Jimenez, Ainara Castellanos-Rubio, Leticia Plaza-Izurieta, Iñaki Irastorza, Xabier Elcoroaristizabal, Amaia Jauregi-Miguel, Tamara Lopez-Euba, Carlos Tutau, Marian M. de Pancorbo, Juan Carlos Vitoria, Jose Ramon Bilbao, Coregulation and modulation of NFκB-related genes in celiac disease: uncovered aspects of gut mucosal inflammation, *Human Molecular Genetics*, Volume 23, Issue 5, 1 March 2014, Pages 1298–1310.

46. Hayden M.S., Ghosh S.. NF-κB in immunobiology, Cell Res., 2011, vol. 21.

47. Stone K.P., Kastin A.J., Pan W.. NFκB is an unexpected major mediator of interleukin-15 signaling in cerebral endothelia, Cell. Physiol. Biochem., 2011, vol. 28.

48. Bjarnason I., Peters T.J.. In vitro determination of small intestinal permeability: demonstration of a persistent defect in patients with coeliac disease, Gut, 1984, vol. 25.

49. Parmar A., Greco D., Venäläinen J., Gentile M., Dukes E., Saavalainen P.. Gene expression profiling of Gliadin effects on intestinal epithelial cells suggests novel non-enzymatic functions of pepsin and trypsin, PLoS One, 2013, vol. 8 pg. e66307.

50. Castellanos-Rubio A., Santin I., Martin-Pagola A., Irastorza I., Castaño L., Vitoria J.C., Bilbao J.R.. Long-term and acute effects of gliadin on small intestine of patients on potentially pathogenic networks in celiac disease, Autoimmunity, 2010, vol. 43 (pg. 131-139).

51. Trynka G., Zhernakova A., Romanos J., Franke L., Hunt K.A., Turner G., Bruinenberg M., Heap G.A., Platteel M., Ryan A.W., et al. Coeliac disease-associated risk variants in TNFAIP3 and REL implicate altered NF-κB signalling, Gut, 2009, vol. 58 (pg. 1078-1083).

52. Sarno M, Lania G, Cuomo M, Nigro F, Passannanti F, Budelli A, Fasano F, Troncone R, Auricchio S, Barone MV, Nigro R, Nanayakkara M. Lactobacillus paracasei CBA L74 interferes with gliadin peptides entrance in Caco-2 cells. Int J Food Sci Nutr. 2014 Dec;65(8):953-9. doi: 10.3109/09637486.2014.940283. Epub 2014 Jul 17. PMID: 25030417

53. Stoven S, Murray JA, Marietta EV. Latest in vitro and in vivo models of celiac disease. Expert Opin Drug Discov. 2013 Apr;8(4):445-57. doi: 10.1517/17460441.2013.761203. Epub 2013 Jan 8. PMID: 23293929; PMCID: PMC3605231.

54. Y. Wang, M. DiSalvo, D.B. Gunasekara, J. Dutton, A. Proctor, M.S. Lebhar, I.A. Williamson, J. Speer, R.L. Howard, N.M. Smiddy, S.J. Bultman, C.E. Sims, S.T. Magness, N.L. Allbritton, Self-renewing Monolayer of Primary Colonic or Rectal Epithelial Cells, Cell Mol Gastroenterol Hepatol 4(1) (2017) 165-182.e7.

55. T. Sato, R.G. Vries, H.J. Snippert, M. van de Wetering, N. Barker, D.E. Stange, J.H. van Es, A. Abo, P. Kujala, P.J. Peters, H. Clevers, Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche, Nature 459(7244) (2009) 262-5.

56. K.L. VanDussen, J.M. Marinshaw, N. Shaikh, H. Miyoshi, C. Moon, P.I. Tarr, M.A. Ciorba, T.S. Stappenbeck, Development of an enhanced human gastrointestinal epithelial culture system to facilitate patient-based assays, Gut 64(6) (2015) 911-20.

57. M. Nanayakkara, G. Lania, M. Maglio, R. Auricchio, C. De Musis, V. Discepolo, E. Miele, B. Jabri, R. Troncone, S. Auricchio, M.V. Barone, P31-43, an undigested gliadin peptide, mimics and enhances the innate immune response to viruses and interferes with endocytic trafficking: a role in celiac disease, Sci Rep 8(1) (2018) 10821.

58. I. Nakagawa, A. Amano, N. Mizushima, A. Yamamoto, H. Yamaguchi, T. Kamimoto, A. Nara, J. Funao, M. Nakata, K. Tsuda, S. Hamada, T. Yoshimori, Autophagy defends cells against invading group A Streptococcus, Science 306(5698) (2004) 1037-40.

59. J.H. Shi, S.C. Sun, Tumor Necrosis Factor Receptor-Associated Factor Regulation of Nuclear Factor κB and Mitogen-Activated Protein Kinase Pathways, Front Immunol 9 (2018) 1849.

60. Salameh, D., Nigro, F., Cante, R. C., Passannanti, F., Gallo, M., Budelli, A., ... & Nigro, R. (2019). Fermentation of rice flour supernatant using Lactobacillus paracasei Cba L74. *Chemical Engineering Transactions*, *75*, 289-294.

61. Sarno M, Lania G, Cuomo M, Nigro F, Passannanti F, Budelli A, Fasano F, Troncone R, Auricchio S, Barone MV, Nigro R, Nanayakkara M. Lactobacillus paracasei CBA L74 interferes with gliadin peptides entrance in Caco-2 cells. Int J Food Sci Nutr. 2014 Dec;65(8):953-9. doi: 10.3109/09637486.2014.940283. Epub 2014 Jul 17. PMID: 25030417.

62. Brodkorb A, Egger L, Alminger M, Alvito P, Assunção R, Ballance S, Bohn T, Bourlieu-Lacanal C, Boutrou R, Carrière F, Clemente A, Corredig M, Dupont D, Dufour C, Edwards C, Golding M, Karakaya S, Kirkhus B, Le Feunteun S, Lesmes U, Macierzanka A, Mackie AR, Martins C, Marze S, McClements DJ, Ménard O, Minekus M, Portmann R, Santos CN, Souchon I, Singh RP, Vegarud GE, Wickham MSJ, Weitschies W, Recio I. INFOGEST static in vitro simulation of gastrointestinal food digestion. Nat Protoc. 2019 Apr;14(4):991-1014. doi: 10.1038/s41596-018-0119-1. Epub 2019 Mar 18. PMID: 30886367.

63. V.S.W. Li, Modelling intestinal inflammation and infection using 'mini-gut' organoids, Nat Rev Gastroenterol Hepatol 18(2) (2021) 89-90.

64. Nanayakkara M, Porpora M, Conte M, Lania G, Bellomo C, Rapacciulo L, Auricchio R, Troncone R, Auricchio S and Barone M V*****. International Journal of Molecular Sciences (submit)

65. Conte, M.; Porpora, M.; Nigro, F.; Nigro, R.; Budelli, A.L.; Barone, M.V.; Nanayakkara, M. Pro-Pre and Postbiotic in Celiac Disease. *Appl.Sci.* 2021, *11*, 8185. https://doi.org/10.3390/app11178185

66. Freire R, Ingano L, Serena G, Cetinbas M, Anselmo A, Sapone A, Sadreyev RI, Fasano A, Senger S. Human gut derived-organoids provide model to study gluten response and effects of microbiota-derived molecules in celiac disease. Sci Rep. 2019 May 7;9(1):7029. doi: 10.1038/s41598-019-43426-w. PMID: 31065051; PMCID: PMC6505524.

67. Caio G, Volta U, Sapone A, Leffler DA, De Giorgio R, Catassi C, Fasano A. Celiac disease: a comprehensive current review. BMC Med. 2019 Jul 23;17(1):142. doi: 10.1186/s12916-019-1380-z. PMID: 31331324; PMCID: PMC6647104.

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Ciclo	**34°**
Codice borsa	**DOT1318210 - Borsa 3**
CUP	**E66C18000940007**
Titolo Progetto	**La gliadina induce l'attivazione del complesso di mTOR e NFK-β in cellule epiteliali intestinali: ruolo del probiotico Lattobacillus Paracasei CBA L74**

La borsa di dottorato è stata cofinanziata con risorse del
Programma Operativo Nazionale Ricerca e Innovazione 2014-2020 (CCI 2014IT16M2OP005),
Fondo Sociale Europeo, Azione I.1 "Dottorati Innovativi con caratterizzazione Industriale"

COMMUNICATIONS BIOLOGY

https://doi.org/10.1038/s42003-020-0906-4 OPEN

Author Correction: Constitutive alterations in vesicular trafficking increase the sensitivity of cells from celiac disease patients to gliadin

Giuliana Lania, Merlin Nanayakkara, Mariantonia Maglio, Renata Auricchio, Monia Porpora, Mariangela Conte, Maria Antonietta De Matteis, Riccardo Rizzo, Alberto Luini, Valentina Discepolo, Riccardo Troncone, Salvatore Auricchio & Maria Vittoria Barone

Correction to: *Communications Biology* https://doi.org/10.1038/s42003-019-0443-1, published online 20 May 2019.

In the original published version of this article the grant number attributed to the Italian Society for Celiac Disease (Associazione Italiana Celiachia; AIC) was incorrectly given as Project No. 053_FC_2013. The correct grant number is Grant 005_FC_2016, awarded to Monia Porpora. The error has been corrected in the HTML and PDF versions of the article.

Published online: 02 April 2020

International Journal of
Molecular Sciences

MDPI

Article

Structural Perspective of Gliadin Peptides Active in Celiac Disease

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Received: 27 November 2020; Accepted: 4 December 2020; Published: 6 December 2020

Abstract: Gluten fragments released in gut of celiac individuals activate the innate or adaptive immune systems. The molecular mechanisms associated with the adaptive response involve a series of immunodominant gluten peptides which are mainly recognized by human leucocyte antigen (HLA)-DQ2.5 and HLA-DQ8. Other peptides, such as A-gliadin P31–43, are not recognized by HLA and trigger innate responses by several routes not yet well detailed. Among the gluten fragments known to be active in Celiac disease, here we focus on the properties of all gluten peptides with known tri-dimensional structure either those locked into HLA-DQ complexes whose crystals were X-ray analyzed or characterized in solution as free forms. The aim of this work was to find the structural reasons why some gluten peptides prompt the adaptive immune systems while others do not, by apparently involving just the innate immune routes. We propose that P31–43 is a non-adaptive prompter because it is not a good ligand for HLA-DQ. Even sharing a similar ability to adopt polyproline II structure with the adaptive ones, the way in which the proline residues are located along the sequence disfavors a productive P31–43-HLA-DQ binding.

Keywords: P31–43; NMR structure; HLA-DQ; innate and adaptive immune systems

1. Introduction

In celiac individuals, the peptides deriving from the digestion of gluten proteins of wheat, barley, oats, and rye cause immune reactions and thus inflammation of the intestinal mucosa. The enzymatic hydrolysis of the hundred proteins contained in the gluten causes the release of peptides in the gut, some of which are able to activate the innate or adaptive immune systems. The aim of this work is to identify the structural characteristics that make different gliadin peptides competent for one or the other of the immune systems.

Up until today, the molecular mechanism associated to the innate immune response to gluten peptides is less detailed than that associated to the adaptive response. Previous research about the chain of pathological events related to Celiac disease (CeD) led to the identification of human leucocyte antigen (HLA) as the molecular player deputed to present the antigenic peptides to T-cells [1]. The interesting history of how HLA was discovered, and who discovered it, was passionately reviewed by E. Thorsby [2].

In CeD, the αb T-cells antigen receptor (TCR) specifically recognizes de-amidated gluten peptide when presented by HLA-DQ2.5 (expressed by 90% of CeD patients), HLA-DQ8 or

HLA-DQ2.2 [3]. Interestingly, the adaptive response is directed versus just few epitopes among the plethora of very similar gluten peptides produced by digestion and resident in the gut [4]. These immunodominant epitopes are HLA-DQ2.5-glia-α1a, DQ2.5-glia-α2, DQ2.5-glia-ω1, DQ2.5-glia-ω2 [5,6] and DQ8-glia-α1 [5,7]. Indeed, other gliadin and glutenin peptides are also involved in CeD immunoreactions. A coherent selection of DQ2.5, DQ2.2, DQ8 and DQ8.5 restricted epitopes recognized by CD4+ T cells and a new nomenclature for this selection was published in 2012 and updated in 2020 by Ludvig M. Sollid [4,8]. The first crystallographic structures of gluten peptide-HLA-DQ and peptide-HLA-DQ/T-cell-receptor, binary and ternary complexes, respectively, were published starting from 2004 (Protein Data Bank (PDB) code 1S9V, [9]) and 2012 (PDB code 4GG6, [7]), respectively. Since then, the X-ray images allowed the structural reasons why HLAs recognize specific peptides and TCRs recognize specific HLA-peptide complex in CeD to be explained. Beside the immunodominant peptides (DQ2.5-glia-α1a, DQ2.5-glia-α2, DQ2.5-glia-ω1, DQ2.5-glia-ω2 and DQ8-glia-α1), a number of gluten fragments are known to provoke inflammation of the intestinal mucosa by stress/innate but not adaptive immune response. Why they are dangerous and how they work in the gut of CeD patients is still not fully deciphered. One of the most investigated peptides belonging to this category is A-gliadin P31–43, L^{31}GQQQPFPPQQPY^{43} [10]. This peptide, not presented by either HLA-DQ2 or HLA-DQ8 [11], activates innate immune response following several routes. A series of studies show that this gliadin peptide is able to activate interferon-α (IFN-α), a mediator of the immune response in the intestine of CeD patients, and an enterocyte cell line, CaCo-2 [12]. In co-operation with a viral ligand, P31–43 is able to interfere with endocytic trafficking thus activating the toll like receptor 7 (TLR7) pathway [12]. This prompted the idea that, together with viral infections, alimentary proteins able to mimic and potentiate the innate immune response to viruses, can trigger an autoimmune disease such as CeD.

Other studies show how P31–43, by binding NBD1 domain of cystic fibrosis trans-membrane conductance regulator (CFTR), an anion channel involved in the epithelial adaptation to environmental stress, impairs CFTR function that in turn generates epithelial stress, tissue transglutaminase and inflammasome activation [13]. Moreover, recent studies propose that the toxic behavior of P31–43 in CeD individuals is due to its ability to self-aggregate and form oligomers able to induce NLRP3 Inflammasome/Caspase 1- dependent mucosal damage in the small intestine [14]. Finally, a very recent study proposes a synergy among tissue transglutaminase TG2, anti-TG2 antibodies and peptide P31–43 in CeD starting and progression [15]. A complete review about the multiple pro-inflammatory effects induced by the gliadin peptide P31–43 is currently in press [16].

Recently, simulated docking experiments suggest that the reason why P31–43 does not work into the adaptive immune circuit is that P31–43 is not a good binder for HLA-DQ, in particular for DQ2 [17]. In the present contribution, we report our analysis of all the known X-ray binary and ternary complexes involving gluten fragments in HLA-DQ and HLA/TCR complexes with the aim to catch, if any, the structural properties that make specific gluten fragments immune-dominant epitopes and some others, like gliadin P31–43, non-ligands.

2. Results

2.1. More Details about the P31–43 Structure Free in Solution

We recently published the structural properties of P31–43 obtained by proton nuclear magnetic resonance (NMR) in solution [17]. This peptide, being very rich in prolines, shows specific conformational properties. Indeed, in peptides and proteins, each peptide bond connecting pairs of adjacent residues generally adopts *trans* configuration (dihedral angle ω of the backbone Cαi − C'i – Ni + 1 – Cαi + 1 atoms is ~180 °) because, due to the smaller steric hindrance between the side chains of the adjacent amino acids, it is on average more stable of about 2.5 kcal mol^{-1} of the *cis* form (ω ~ 0 °) [18,19]. The *trans* isomer is also kinetically stabilized in reason of an isomerization barrier *trans* → *cis* of about 20 kcal mol^{-1} [18]. However, in the Xxx-Pro bond (Xxx = any residue),

frequently found in gluten peptides, the *trans* and *cis* conformations which only differ by ~0.5 kcal mol^{-1} are practically iso-energetic, and show inter-conversion barrier as low as 13 kcal mol^{-1} [19]. This means that while in proteins the three-dimensional structure forces the Xxx-Pro bond mainly in *trans* configuration (*cis* configuration is observed in just ~5 % of cases [19,20]), in peptides the Xxx-Pro bond visits both the configurations. Thus, while in peptides with no Pro residues all peptide bonds are in *trans* configuration (in the following 'all *trans*'), Pro bearing peptides may adopt *trans* and *cis* configuration at each Xxx-Pro bond in the sequence. Other relevant issue for peptides that, as gluten fragments, contain Pro, is that the cyclic structure of proline's side chain locks the proline φ angle at approximately -65° [21], a value that favors polyproline II structure (PPII: $\varphi \sim -75^{\circ}$, $\psi \sim 150^{\circ}$).

How peptide P31–43 works in CeD depends on its structure and on the structure of its molecular targets. P31–43 contains 13 residues including 4 prolines. This means that it can potentially adopt 4^2 different structures due to the different *cis-trans* combinations of the four Xxx-Pro bonds.

The structural differences between the numerous forms are relevant, as can be appreciated in Figure 1, where P31–43 modelled with all the four prolines 'in *trans*' or Pro36, Pro38, and Pro39 singularly in *cis* configuration, is shown. Still, other structures may come from *cis* Pro42 and all possible combinations of two, three or all four *cis* bonds.

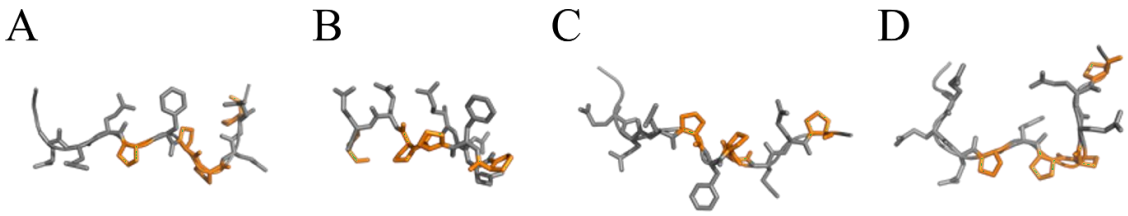

Figure 1. P31–43 (L^{31}GQQQPFPPQQPY^{43}) modelled in (**A**) 'all *trans*', (**B**) *cis* Gln35-Pro36, (**C**) *cis* Phe37-Pro38, and (**D**) *cis* Pro38-Pro39 configurations. Proline residues are colored in gold.

P31–43 conformational behavior in solution has been investigated in recent times by proton nuclear magnetic resonance [17], circular dichroism, fluorescence spectroscopy, several techniques characterizing self-aggregate forms of the peptide and molecular dynamics studies in oligomeric and monomeric forms of the peptide [14,22,23].

The conformational analysis of P31–43 in aqueous solution performed through NMR [17] shows that, although the sequence contains a single Phe and a single Tyr residue, each of these residues exhibits well more than two spin systems, indicating that Phe and Tyr are not only affected by the different *cis*/*trans* configurations of the peptide bond in which they are involved but also by the different configuration of the other Xxx-Pro bonds further away in the sequence. This phenomenon can be observed in the expansion of the reported Tocsy scalar correlation spectrum of P31–43 reported in Figure 2.

The complexity of the two-dimensional NMR proton spectra confirmed that the peptide adopts a variety of *cis*/*trans* combinations of Xxx-Pro bonds, potentially all 4^2 theoretically predictable structures with different percentages and in slow inter-conversion kinetic at the NMR time scale. The analysis allows the identification of the 'all *trans*' structure and to describe its conformational propensities. Starting from measurements of NOE dipolar coupling effects, NMR analysis allows the time averaged distances between pairs of hydrogen nuclei placed along the sequence to be estimated. Once the inter-protons distances (NMR distances) are determined, they are used as experimental constraints for calculating a number of peptide structures compatible with them. The greater the number of the experimental constraints, the more accurately the secondary structure of the peptide is defined. The structures that best adhere to the NMR distances, taken all together, represent the conformational behavior of the peptide in solution. A way to catch how wide the distribution of conformations is, is to group the structures by similarity. The higher the number of clusters, the wider the structures distribution is and the higher the peptide flexibility is. Moreover, the higher the population of a single

cluster, the higher the weight of that structure is in describing the conformational preferences of the peptide. The forty P31–43 structures obtained by NMR analysis (deposited with PDB code 6QAX), once clustered by resemblance, revealed six different groups of which the first three most populated ones, counting for more than 50% of the entire structure population, are shown in Figure 3.

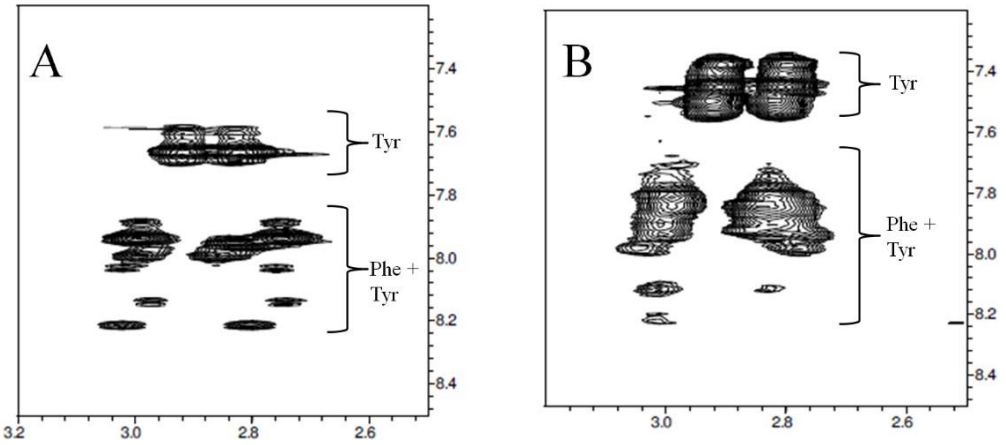

Figure 2. (**A**) Expansion of the Tocsy map of P31–43 in water showing the NH-ββ'CH2 scalar correlation peaks of Phe36 and Tyr43 residues. (**B**) Same map expansion of the Tocsy experiment performed for P31–43 in SDS 150 mM.

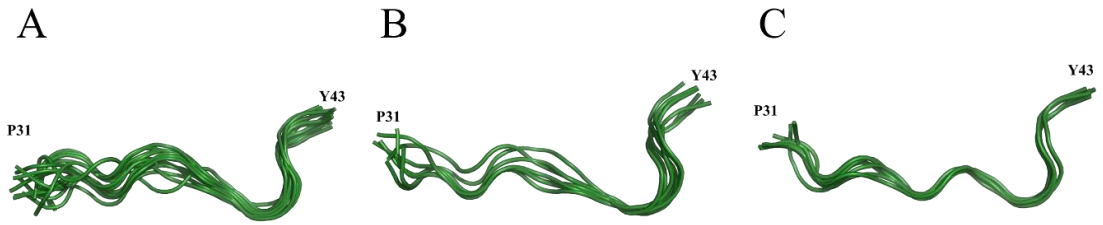

Figure 3. First three clusters of P31–43 NMR structures (ribbon representation) as clustered by Chimera software [24]. (**A**) Cluster 0, the most populated cluster collecting 14 structures over forty; (**B**) cluster 1 with 7 structures over forty; (**C**) cluster 2 with 7 structures over forty.

It is interesting to note that none of the NMR structures showed PPII torsion angles along the entire sequence. Nevertheless, the propensity to adopt this motif is clear in each cluster, particularly in cluster 0 (Figure 3A), where segments of 2–3 consecutive amino acids, differently localized in different structures, adopt PPII conformation. This result is in accordance with circular dichroism measurements showing that the peptide tends to adopt a PPII structure in equilibrium with random structures [14,23]. The peptides that enter or are generated in cells or tissues interact with membranes, receptors, and other molecules, triggering processes with a favorable or unfavorable outcome for the host organism. To characterize the ability of P31–43 to interact with biological membranes, the peptide was incubated with SDS, a membrane mimic, and the interaction tested by fluorescence analysis [25]. This same interaction was also tested by using the NMR technique [17]. Although SDS micelles do not represent the best system for mimicking human cell membranes, they have been used to verify whether the interaction with a micellar surface had the ability to restrict the variety of conformations exhibited by P31–43 in free form. The spectrum shown in Figure 2B shows wider peaks with respect to those visible in the analogous spectrum of Figure 2A. The widening of the peaks confirms the interaction between peptide and SDS, since it indicates that the peptide tumbles in solution at lower frequencies than those of the free peptide, that is it rolls at the tumbling frequencies of the micelles to which it adheres. Nevertheless, the fact that the number of signals is not significantly reduced

(compare Figure 2A,B) indicates that the micelles formed by SDS are not able to stabilize particular conformations of P31–43 and that ultimately the peptide-SDS interaction is non-specific.

2.2. Structure of Gluten Peptides Able to Bind or Not in the HLA-DQ Groove

The physiological manifestations linked to CeD are partly due to the activation by some gliadin peptides of the gluten-reactive CD4+ T-cells of the intestinal mucosa. The molecular mechanism is known and foresees that (i) the gliadin peptide is recognized and deamidated to one of its Gln by TG2 [26]; (ii) the deamidation transforms the gluten peptide in a better binder for HLA-DQ [27]; (iii) the deamidated peptide is recognized mainly by the type II HLA-DQ2 or DQ8 expressed by celiac subjects [28,29]; (iv) the complex is recognized by the CD4+ T cell with consequent induction of proliferative effects.

The first crystallographic structure of the complex formed by the soluble portion of HLA-DQ2 and the gliadin peptide α1 (DQ2-glia-α1 = PFPQPE^{6}LPY, nomenclature of gliadin epitope following Sollid et al. [8]) was published by Ludvig M. Sollid and co-authors in 2004 [9]. HLA-DQ2 general folding is typical of the MHC class II family which HLA belongs to. The groove that houses the peptide α1 has a typical architecture formed by a β-sheet platform on which two parallel α-helices sit on as walls (Figure 4), while the peptide glia-α1 adopts a conformation close to PPII (all *trans* peptide bonds, $\varphi \approx -75^{\circ}$, $\psi \approx 150^{\circ}$). It must be noted that having four proline residues, the α1 peptide lacks as many as 4 NH amides, possibly useful for establishing hydrogen bonds with the groove. Nevertheless, as underlined by Chu-Young Kim and co-authors [9], α1 peptide docks into the binding pocket in a way (i.e., register) that is the best one for maximizing the network of H-bonds (particularly relevant to those involving Glu6 side chain), that together with charge and hydrophobic interactions make the complex stable (see Figure 3 of [9]).

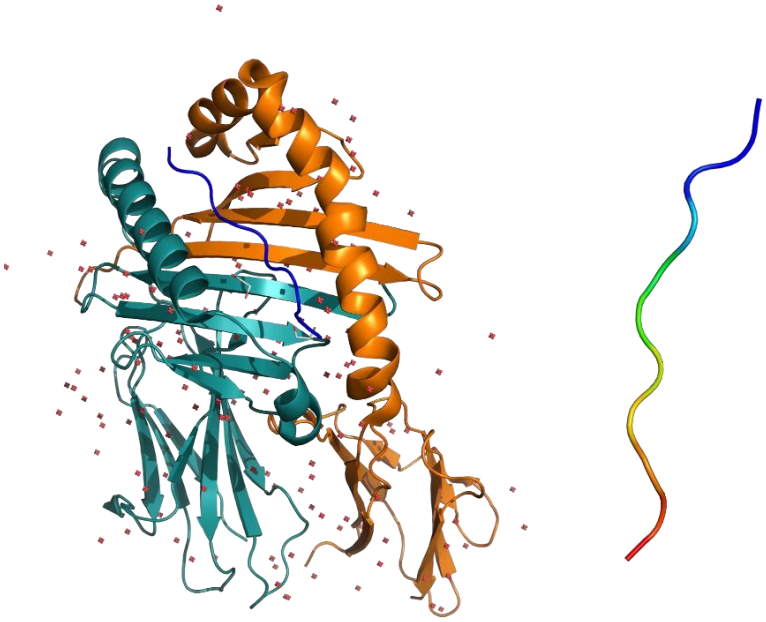

Figure 4. (Left) X-ray structure of HLA-DQ2.5-glia-α1 complex (PDB code 1S9V); (**Right**) glia-α1 structure as found in the complex (color code: blue = N-terminal end, red = C-terminal end.).

Different HLA-DQs show different specificity for different gluten peptides because of the diversity of amino acid side-chains anchoring the peptide into the groove.

The list of PDB structures of binary and ternary complexes involving HLA-DQ, gluten peptides and T-cell receptor updated to October 2020 is reported in Table 1.

Table 1. The list of Protein Data Bank (PDB) structures of binary and ternary complexes involving human leucocyte antigen (HLA)DQ, gluten peptides and T-cell receptor (TCR) updated to October 2020.

PDB ID	HLA-II	Binder	Complex	References
6U3M	DQ2.5	mimic glia- α1a	binary	[30]
6U3N	DQ2.5	mimic glia- α1a	ternary (TCR LS2.8/3.15)	[30]
6U3O	DQ2.5	mimic glia- α2a	ternary (TCR JR5.1)	[30]
6PY2	DQ2.2	glut-L1	ternary (TCR T594)	[31]
6MFF	DQ2.5	glia-ω1	binary	[5]
6MFG	DQ2.5	glia-α1a	binary	[5]
4OZI	DQ2.5	glia-α1a	ternary (TCR S2)	[1]
1S9V	DQ2	α1	binary	[9]
4OZF	DQ2	α2	ternary (TCR JR51)	[1]
4OZG	DQ2	α2	ternary (TCR d2)	[1]
4OZH	DQ2	α2	ternary (TCR s16)	[1]
5KS9	DQ8	glia-α1	ternary (Bel502 TCR)	[32]
5KSA	DQ8.5	glia-γ1	ternary (Bel602 TCR)	[32]
5KSB	DQ8.5	glia-γ1	ternary (T15 TCR)	[32]
4Z7U	DQ8	glia-α1	ternary (S13 TCR)	[33]
4Z7V	DQ8	glia-α1	ternary (L3 − 12 TCR)	[33]
4Z7W	DQ8	glia-α1	ternary (T316 TCR)	[33]
4GG6	DQ8	glia-α1	ternary (SP3.4 TCR)	[7]
2NNA	DQ8	glia-α1	binary	[34]
4D8P	DQ2.3	peptide	binary	[35]

Peptide binding register of immunodominant peptides in these complexes with HLA-DQ and T cell receptor is shown in Figure 5.

	Peptide-binding register												
Epitopes	**P-3**	**P-2**	**P-1**	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**	**P10**	
DQ2.5–P.fuor-α1a				P	M	P	M	P	E	L	P	Y	P	6U3M
DQ2.5–P.fuor-α1a				P	M	P	M	P	E	L	P	Y	P	6U3N
DQ2.5-glia-α1a	Q	L	Q	P	F	P	Q	P	E	L	P	Y		6MFG
DQ2.5-glia-ω1	P	Q	Q	P	F	P	Q	P	E	Q	P	F	P	6MFF
DQ2.5-glia-α1a				P	F	P	Q	P	E	L	P	Y		4OZI
DQ2 α1		L	Q	P	F	P	Q	P	E	L	P	Y		1S9V
DQ2.5–P.acru-α2a				M	V	V	Q	S	E	L	P	Y	PE	6U3O
DQ2α2						P	Q	P	E	L	P	Y	PQP	4OZF, 4OZG, 4OZH
DQ8-glia-α1	P	S	G	E	G	S	F	Q	P	S	Q	E	NPQ[12]	5KS9
DQ8-glia-α1		S	G	E	G	S	F	Q	P	S	Q	E	NP	4Z7U 4Z7V,4Z7W
DQ8-glia-α1		S	G	E	G	S	F	Q	P	S	Q	E	NP[11]	4GG6
DQ8-glia-α1 QQY	P	S	G	E	G	S	F	Q	P	S	Q	E	NPQ[12]	2NNA
DQ8.5-glia-γ1			Q	P	Q	Q	S	F	P	E	Q	E		5KSA, 5KSB
DQ2.2-glut-L1				P	F	S	E	Q	E	Q	P	V	L	6PY2
DQ2.3-pcpt	P	Q	P	E	Q	P	E	Q	P	F	P	Q	P	4D8P

Figure 5. Peptide binding register of immunodominant peptides in complexes with HLA-DQ and T-cell receptor.

We isolated the peptide ligands from the complexes and compared them with the aim to find, if any, the structural properties that make specific gluten fragments immunodominant epitopes and some others, like gliadin P31–43, non-ligands. The superimposition of four DQ2.5-glia-α1 X-ray structures is shown in Figure 6. Even though belonging to different data collections, the α1 conformation in the HLA-DQ2.5 groove is strictly reproduced. Some structural differences are just found in the orientation of Phe and Tyr side chains in P2 and P9 sites of the DQ2.5 binding pocket, respectively. In the bundle in Figure 5, we also included DQ2.5-glia-ω1 (colored in orange). This gliadin epitope differs from α1 for having Leu instead of Gln at P7 and Phe instead of Tyr at P9 (further than two

other less relevant differences at P-3 and P-1 sites, see Figure 5). A visual comparison shows that four diverse residues do not change the glia-ω1 backbone conformation respect to that of glia-α1. Very recently, Petersen et al. [30] published the X-ray structures of HLA-DQ2.5 complex to peptide fragments from bacteria proteins with high sequence homology with glia-α1 and α2. The gliadin mimic from *Pseudomonas fluorescence*, DQ2.5–*P.fluor*-α1a, differs from glia-α1a for having two Met residues instead of Phe and Gln in P2 and P4 sites. As it can be seen in Figure 6, the mimic peptide, represented as blue sticks, adopts the backbone structure similar to those adopted by glia-α1 ligands and glia-ω1. The comparison in Figure 6 demonstrates that the topology of the binding groove shapes all ligands in a PPII-like conformation. The quantitative measures of the similarity between superimposed atomic coordinates of the peptides, i.e., the root mean square deviation (RMSD) values, are listed in Table S1 of the Supplementary Materials.

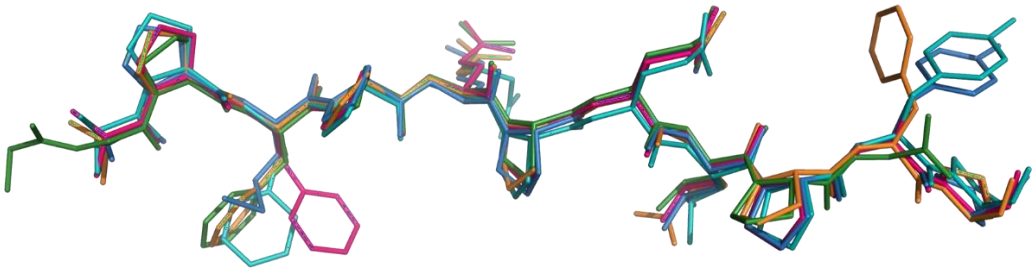

Figure 6. Superimposition of DQ2α1 (from binary complex, PDB code 1S9V, forest), DQ2.5-glia-α1a (from binary complex, PDB code 6MFG, hot pink) DQ2.5-glia-α1a (from ternary complex, PDB code 4OZI, teal), DQ2.5-glia-ω1 (from ternary complex, PDB code 6MMF, orange), DQ2.5–*P.fluor*-α1a (from binary complex, PDB code 6U3M, blue).

P31–43 is known not to be a ligand either for HLA-DQ2.5 or DQ8 [11]. Moreover, the in silico experiments performed by docking P31–43 onto HLA-DQ2.5 show that the peptide in the experimental structure, as well as modelled in PPII, engages in both conditions a number of interactions with the binding groove lower than that established by glia-α1 [17]. A representation of such finding is visible in Figure 7 where DQ2.5 glia-α1 and P31–43 in PPII structure are superimposed. In this case, the accordance between the two peptides shows an RMSD value of 1.30 Å, well above the RMSD values calculated between DQ2.5-glia-α1a and DQ2.5–*P.fluor*-α1a or between Q2.5-glia-α1a and DQ2.5-glia-ω1 (Table S1). The PPII arrangement of P31–43 is not enough for a correct positioning of Pro residues into the groove because, even if P31–43 bears the same number of Pro as in DQ2.5-glia-α1, those are differently localized along the sequence. The consequence is that P31–43, differently from glia-α1, does not find any useful register to establish the network of H-bonds needed to stabilize the complex.

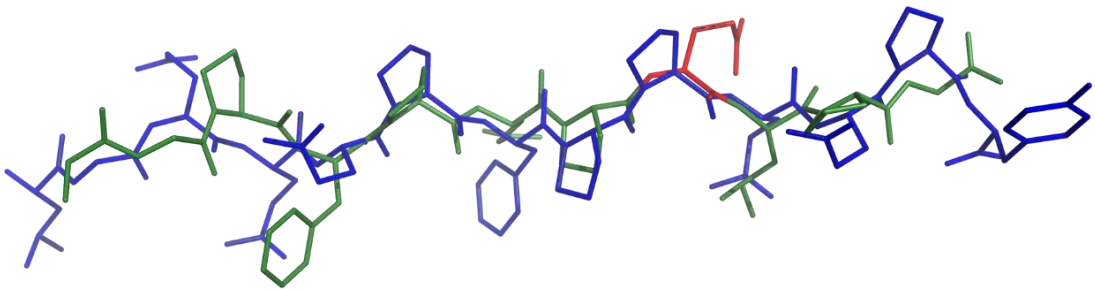

Figure 7. Superimposition of DQ2-α1 (from binary complex PDB code 1S9V, colored in forest with its glutamic acid at P6 colored in red) with P31–43 modelled in PPII structure (blue).

The gluten peptides that bind to HLA-DQ8 are different from those recognized by HLA-DQ2.5. Particularly, DQ8 epitopes contain no more than two prolines in the binding core in respect to the

three-four Pro residues of HLA-DQ2.5 core epitopes and, importantly for the binding, Glu residues at P1 and P9 sites.

The superimposition of DQ8-glia-α1 peptides, as extracted from thebinary and ternary X-ray complexes, shows a strict reproducibility among the ligand structures. HLA-DQ8 binding groove shapes the ligand in a PPII-like conformation. Again, to catch the differences in binding ability between DQ8-glia-α1 and P31–43, we consider the superimposition of the first with the second modelled in PPII (RMSD 1.1 Å). As it can be observed in Figure 8, P31–43 (blue sticks) shows residue side-chains totally different both for nature and localization from those of DQ8-glia-α1 (green), thus failing in stabilizing the right interactions with the groove.

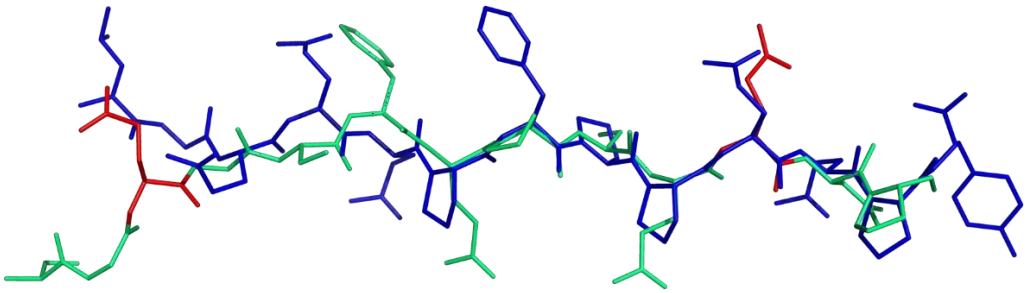

Figure 8. Superposition of DQ8-glia- α1 (from binary complex PDB code 2NNA, colored in green with Glu residues at P1 and P9 colored in red) with P31–43 modelled in PPII structure (blue).

3. Discussion

Here we report the analysis of all structurally characterized gluten fragments active in CeD with the objective to find the structural reasons that make specific gluten fragments players in the immune-adaptive system by binding HLA-DQs molecules, and some others, like gliadin P31–43, non-ligands of HLA, and instead players in innate immune routes.

The immunodominant gliadin peptides productively interact and bind type II HLA receptors because the process is energetically favored. Differently, P31–43, very similar by composition to the immunodominant gliadin peptides, does not bind type II HLAs, meaning that for some reasons the binding process is disfavored. In order to understand why, we considered comparing the structural properties of the peptides belonging to both categories to be useful, that is the binders and not-binders of HLA-DQ. The NMR study of P31–43 in solution shows that the peptide exhibits *cis*/*trans* isomerism at Xxx-Pro sites and, for each isomer, the peptide adopts a distribution of different conformers. The 'all *trans*' structures represent at least 60–65% of the entire population and exhibit a clear tendency to adopt PPII conformation [17], i.e., that 'required' by HLA binding. Dynamic light scattering (DLS) measurements showed that P31–43 forms aggregates in solution [14]. For that, by considering NMR and DLS results together, the emerging picture is that P31–43 lives in solution distributed between monomeric and oligomeric forms. By assuming a model of pseudo-equilibrium like monomers ↔ aggregates, NMR describes the conformational preferences of the monomeric entity, and in the case of fast exchange regime of the monomer with the NMR transparent large aggregates, the technique reads the conformational tendency of the monomer mixed to its memoire of the structure adopted into the aggregates [36]. Barrera and co-authors, in a very recent commentary, re-analyzed their dynamic simulation studies of P31–43 and found 'remarkable agreement' between experimental NMR structures and those visited by the monomer during the simulations as well as those adopted in the simulated oligomeric forms, thus, suggesting "that P31–43 suffers very minor conformational changes when passing from monomeric to oligomeric states" [22].

This study deals with the structural comparison among P31–43 and the immunodominant peptides (Table 1). With the exception of DQ2α2 (PQPELPY), of which a strict analogue (PQPQLPY) was characterized in free form by solution NMR [37], all the others are structurally known only in complex with HLA-DQ. Because containing Pro residues, all those peptides are expected to exhibit in free form

the same *cis*/*trans* isomerism and conformers distribution as found for P31–43 in solution. They are flexible enough to adapt to the binding pocket and at the same time rigid enough, thanks to the prolines, to minimize the negative entropic variation associated with the binding. When bound to HLA-DQ, they all adopt a PPII-like structure inside the binding pocket. That appears as an interesting property of the groove topology that, shared by different HLA-DQs, forces different gliadin epitopes into a strictly similar conformation. Indeed, the backbone superimpositions among the gliadin ligands show very low RMSD values ranging from 0.178 to 0.425 Å, if we consider for example the DQ2.5-glia-α vs a series of other DQ2.5 epitopes (Table S1). When the docking of P31–43 into HLA-DQ2.5 was simulated, it was found that the peptide, both in the experimental structure or modelled in PPII, engages with the groove causing a number of interactions sensibly lower than those established by the DQ2.5-glia-α1 [17]. P31–43 shows an arrangement of its prolines different from that of DQ2.5-glia-α1 and this plays against the binding with HLADQ2.5. P31–43 does not appear to be a good binder for HLA-DQ8 either. By sequence and amino acid composition, P31-43 is very different from the epitopes of DQ8 and therefore unsuitable to engage the interactions specified by HLA-DQ8 binding groove.

While the structural reasons why P31–43 is not an adaptive immune player via HLA-DQ molecules are apparently deciphered, those underlining the many mechanisms in which it is involved as innate immune agent remain to be clarified. Gomez-Castro and co-authors [14] propose that P31–43, due to its ability to self-aggregate, acts at oligomeric state in triggering the NPRP3 inflammosome and thus the intestinal pathology. Herrera and co-authors [23] suggest that the formation of P31–43 nanostructures induces proinflammatory effects and subsequent damage at the intestinal mucosa in CeD. We think that last decades of experimental and simulative works made clear that all peptides adopt a distribution of conformations in solution or fluid media, that in dependence of the concentration, they participate to apparent equilibria with ordered/un-ordered aggregates floating with them in the common media, and that, opportunely treated, all peptides can 'solidify' ordinately in the form best adhering to their nature, i.e., α or β-fibers.

Although the observation that the toxic behavior of P31–43 could be due to its self-aggregation ability represents an interesting model, we observe that knowing in which proportion the peptide is distributed among the various oligomeric forms depends upon the environments and this is an issue difficult to be estimated in cells. Indeed, we suggest that P31–43, together with almost all other protein fragments, represent objects that may act in several ways following several routes in reason of the cell district they enter, the concentration they are able to reach there, and the molecular or supramolecular entities they come into contact with.

In conclusion, it is interesting to note that starting from gliadin proteins, known to be very monotonous macromolecules, made up from blocks of similar sequences repeated many times, the enzymatic digestion releases in gut fragments that provoke different biological responses in CeD individuals [16]. P31–43, a not adaptive immune player, shares a similar content in proline residues, similar adaptability to polyproline II structure, but a different positioning of the proline residues along the sequence with the adaptive ones. This last issue disfavors a productive binding to HLA-DQ2.5 and thus P31–43 access to the adaptive immune route.

4. Materials and Methods

4.1. NMR Analysis

P31–43 peptide was purchased from Inbios (Naples, Italy). Deuterated solvents, such as D2O, (99,8% isotopic purity) and sodium dodecyl-d_{25} sulphate (SDS-d25, > 98 atom % D) were purchased from Sigma-Aldrich (Milan, Italy). NMR measurements of P31–43 in aqueous solution were obtained at 600MHz proton resonance frequency as previously reported [17]. Briefly, NMR characterizations of P31–43 were performed at 298 K in a H_2O/D_2O 90:10 (*v*/*v*) mixture and in sodium dodecyl sulfate 150 mM (SDS) at pH 4.6 ± 0.1 where the peptide has a net electrical charge equal to zero. Proton resonance assignments were obtained by analyzing the suite of bi-dimensional DQFCOSY,

TOCSY, NOESY and ROESY experiments. Proton assignments of the amino acid spin systems and 1D proton spectrum of P31–43 in aqueous as well as in SDS media are reported [17]. The percentage of P31–43 in 'all *trans*' structure was estimated at about 60–65% of the total population in water solution. The three-dimensional models were obtained by following the classical protocol consisting of: Assignment of the proton chemical shifts, integration of the dipolar effects (NOE), conversion of NOE intensities into inter-proton distances, and calculation of peptide structures compatible with the entire set of distance restraints by using CYANA software [38]. In order to characterize peptide flexibility and weight of different conformations, CYANA structures were clustered by similarity using UCSF Chimera program (version 1.14) [24].

4.2. Structure Comparison of Gluten Peptides

All the X-ray structures of binary HLA-DQ/gluten peptides and ternary HLA-DQ/gluten peptides/T-cell receptor complexes published on the PDB databank on October 2020 (Table 1) were downloaded and analyzed. Particularly, all the gluten fragments inside HLA-DQ grooves were extracted and structurally compared each other. First comparisons consider the differences among the structures of the same sequence in the same HLA-DQX, then of the same sequence in different HLA-DQ. Then, the structural differences between different gliadin ligands were analyzed. Finally, structural differences between those and related bacteria peptide mimetic were tested. All structure comparisons were obtained by peptide superimpositions based on a principle of register correspondence by using PyMOL software (http://www.PyMOL.org) and the open source software MOLMOL 2K.2.0 [39].

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/23/9301/s1.

Author Contributions: Methodology—preparation, binding experiments, M.C., M.N.; methodology—N.M.R. experiments, computational experiments, data analysis, L.C., L.F. and G.D.; writing—original draft preparation, G.D., L.F., L.C. and M.V.B.; writing-review and editing, G.D., L.F.and M.V.B.; conceptualization, G.D., L.F. and M.V.B.; supervision, M.V.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

CeD	Celiac disease
HLA	Human leucocyte antigen
TCR	T-cells antigen receptor
TG	Transglutaminase
PPII	Polyproline II

References

1. Petersen, J.; Montserrat, V.; Mujico, J.R.; Loh, K.L.; Beringer, D.X.; Van Lummel, M.; Thompson, A.; Mearin, M.L.; Schweizer, J.; Kooy-Winkelaar, Y.; et al. T-cell receptor recognition of HLA-DQ2–gliadin complexes associated with celiac disease. *Nat. Struct. Mol. Biol.* **2014**, *21*, 480–488. [CrossRef] [PubMed]
2. Thorsby, E. A short history of HLA. *Tissue Antigens* **2009**, *74*, 101–116. [CrossRef] [PubMed]
3. Sollid, L.M.; Jabri, B. Triggers and drivers of autoimmunity: Lessons from coeliac disease. *Nat. Rev. Immunol.* **2013**, *13*, 294–302. [CrossRef] [PubMed]
4. Sollid, L.M.; Qiao, S.-W.; Anderson, R.P.; Gianfrani, C.; Koning, F. Nomenclature and listing of celiac disease relevant gluten T-cell epitopes restricted by HLA-DQ molecules. *Immunogenetics* **2012**, *64*, 455–460. [CrossRef]
5. Dahal-Koirala, S.; Ciacchi, L.; Petersen, J.; Risnes, L.F.; Neumann, R.S.; Christophersen, A.; Lundin, K.E.A.; Reid, H.H.; Qiao, S.-W.; Rossjohn, J.; et al. Discriminative T-cell receptor recognition of highly homologous HLA-DQ2–bound gluten epitopes. *J. Biol. Chem.* **2019**, *294*, 941–952. [CrossRef]

6. Tye-Din, J.A.; Stewart, J.A.; Dromey, J.A.; Beissbarth, T.; Van Heel, D.A.; Tatham, A.; Henderson, K.; Mannering, S.I.; Gianfrani, C.; Jewell, D.P.; et al. Comprehensive, Quantitative Mapping of T Cell Epitopes in Gluten in Celiac Disease. *Sci. Transl. Med.* **2010**, *2*, 41ra51. [CrossRef]
7. Broughton, S.E.; Petersen, J.; Theodossis, A.; Scally, S.W.; Loh, K.L.; Thompson, A.; Van Bergen, J.; Kooy-Winkelaar, Y.; Henderson, K.N.; Beddoe, T.; et al. Biased T Cell Receptor Usage Directed against Human Leukocyte Antigen DQ8-Restricted Gliadin Peptides Is Associated with Celiac Disease. *Immunity* **2012**, *37*, 611–621. [CrossRef]
8. Sollid, L.M.; Tye-Din, J.A.; Qiao, S.-W.; Anderson, R.P.; Gianfrani, C.; Koning, F. Update 2020: Nomenclature and listing of celiac disease–relevant gluten epitopes recognized by CD4+ T cells. *Immunogenetics* **2019**, *72*, 85–88. [CrossRef]
9. Kim, C.-Y.; Quarsten, H.; Bergseng, E.; Khosla, C.; Sollid, L.M. Structural basis for HLA-DQ2-mediated presentation of gluten epitopes in celiac disease. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 4175–4179. [CrossRef]
10. Maiuri, L.; Ciacci, C.; Ricciardelli, I.; Vacca, L.; Raia, V.; Auricchio, S.; Picard, J.; Osman, M.; Quaratino, S.; Londei, M. Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. *Lancet* **2003**, *362*, 30–37. [CrossRef]
11. Zimmer, K.-P.; Fischer, I.; Mothes, T.; Weissen-Plenz, G.; Schmitz, M.; Wieser, H.; Büning, J.; Lerch, M.M.; Ciclitira, P.C.; Weber, P.; et al. Endocytotic segregation of gliadin peptide 31-49 in enterocytes. *Gut* **2010**, *59*, 300–310. [CrossRef] [PubMed]
12. Nanayakkara, M.; Lania, G.; Maglio, M.; Auricchio, R.; De Musis, C.; Discepolo, V.; Miele, E.; Jabri, B.; Troncone, R.; Auricchio, S.; et al. P31-43, an undigested gliadin peptide, mimics and enhances the innate immune response to viruses and interferes with endocytic trafficking: A role in celiac disease. *Sci. Rep.* **2018**, *8*, 1–12. [CrossRef] [PubMed]
13. Villella, V.R.; Venerando, A.; Cozza, G.; Esposito, S.; Ferrari, E.; Monzani, R.; Spinella, M.C.; Oikonomou, V.; Renga, G.; Tosco, A.; et al. A pathogenic role for cystic fibrosis transmembrane conductance regulator in celiac disease. *EMBO J.* **2019**, *38*, e100101. [CrossRef] [PubMed]
14. Gomez Castro, M.F.; Miculan, E.; Herrera, M.G.; Ruera, C.; Perez, F.; Prieto, E.D.; Barrera, E.; Pantano, S.; Carasi, P.; Chirdo, F.G. Gliadin Peptide Forms Oligomers and Induces NLRP3 Inflammasome/Caspase 1-Dependent Mucosal Damage in Small Intestine. *Front. Immunol.* **2019**, *10*, 31. [CrossRef]
15. Martucciello, S.; Sposito, S.; Esposito, C.; Paolella, G.; Caputo, I. Interplay between Type 2 Transglutaminase (TG2), Gliadin Peptide 31-43 and Anti-TG2 Antibodies in Celiac Disease. *Int. J. Mol. Sci.* **2020**, *21*, 3673. [CrossRef] [PubMed]
16. Chirdo, F.G.; Auricchio, S.; Troncone, R.; Barone, M.V. The gliadin p31-43 peptide: Inducer of multiple proinflammatory effects. *Int. Rev. Cell Mol. Biol.* **2020**, (in press). [CrossRef]
17. Calvanese, L.; Nanayakkara, M.; Aitoro, R.; Sanseverino, M.; Tornesello, A.L.; Falcigno, L.; D'Auria, G.; Barone, M.V. Structural insights on P31-43, a gliadin peptide able to promote an innate but not an adaptive response in celiac disease. *J. Pept. Sci.* **2019**, *25*, e3161. [CrossRef]
18. Ramachandran, G.N.; Sasisekharan, V. Conformation of polypeptides and proteins. *Adv. Protein. Chem.* **1968**, *23*, 283–438.
19. Stewart, D.E.; Sarkar, A.; Wampler, J.E. Occurrence and role of cis peptide bonds in protein structures. *J. Mol. Biol.* **1990**, *214*, 253–260. [CrossRef]
20. MacArthur, M.W.; Thornton, J.M. Influence of proline residues on protein conformation. *J. Mol. Biol.* **1991**, *218*, 397–412. [CrossRef]
21. Morris, A.L.; MacArthur, M.W.; Hutchinson, E.G.; Thornton, J.M. Stereochemical quality of protein structure coordinates. *Proteins Struct. Funct. Bioinform.* **1992**, *12*, 345–364. [CrossRef] [PubMed]
22. Barrera, E.; Chirdo, F.; Pantano, S. Commentary: p31-43 Gliadin Peptide Forms Oligomers and Induces NLRP3 Inflammasome/Caspase 1- Dependent Mucosal Damage in Small Intestine. *Front. Immunol.* **2019**, *10*, 2792. [CrossRef] [PubMed]
23. Herrera, M.G.; Castro, M.F.G.; Prieto, E.; Barrera, E.; Dodero, V.I.; Pantano, S.; Chirdo, F.G. Structural conformation and self-assembly process of p31-43 gliadin peptide in aqueous solution. Implications for celiac disease. *FEBS J.* **2019**, *287*, 2134–2149. [CrossRef] [PubMed]
24. Pettersen, E.F.; Goddard, T.D.; Huang, C.C.; Couch, G.S.; Greenblatt, D.M.; Meng, E.C.; Ferrin, T.E. UCSF Chimera—A visualization system for exploratory research and analysis. *J. Comput. Chem.* **2004**, *25*, 1605–1612. [CrossRef]

25. Vilasi, S.; Sirangelo, I.; Irace, G.; Caputo, I.; Barone, M.V.; Esposito, C.; Ragone, R. Interaction of 'toxic' and 'immunogenic' A-gliadin peptides with a membrane-mimetic environment. *J. Mol. Recognit.* **2010**, *23*, 322–328. [CrossRef]
26. Sollid, L.M. Coeliac disease: Dissecting a complex inflammatory disorder. *Nat. Rev. Immunol.* **2002**, *2*, 647–655. [CrossRef]
27. Arentz-Hansen, H.; Körner, R.; Molberg, Ø.; Quarsten, H.; Vader, W.; Kooy, Y.M.; Lundin, K.E.; Koning, F.; Roepstorff, P.; Sollid, L.M.; et al. The Intestinal T Cell Response to α-Gliadin in Adult Celiac Disease Is Focused on a Single Deamidated Glutamine Targeted by Tissue Transglutaminase. *J. Exp. Med.* **2000**, *191*, 603–612. [CrossRef]
28. Lundin, K.E.; Gjertsen, H.A.; Scott, H.; Sollid, L.M.; Thorsby, E. Function of DQ2 and DQ8 as HLA susceptibility molecules in celiac disease. *Hum. Immunol.* **1994**, *41*, 24–27. [CrossRef]
29. Lundin, K.E.; Scott, H.; Hansen, T.; Paulsen, G.; Halstensen, T.S.; Fausa, O.; Thorsby, E.; Sollid, L.M. Gliadin-specific, HLA-DQ(alpha 1*0501,beta 1*0201) restricted T cells isolated from the small intestinal mucosa of celiac disease patients. *J. Exp. Med.* **1993**, *178*, 187–196. [CrossRef]
30. Petersen, J.; Ciacchi, L.; Tran, M.T.; Loh, K.L.; Kooy-Winkelaar, Y.; Croft, N.P.; Hardy, M.Y.; Chen, Z.; McCluskey, J.; Anderson, R.P.; et al. T cell receptor cross-reactivity between gliadin and bacterial peptides in celiac disease. *Nat. Struct. Mol. Biol.* **2019**, *27*, 49–61. [CrossRef]
31. Ting, Y.T.; Dahal-Koirala, S.; Kim, H.S.K.; Qiao, S.-W.; Neumann, R.S.; Lundin, K.E.A.; Petersen, J.; Reid, H.H.; Sollid, L.M.; Rossjohn, J. A molecular basis for the T cell response in HLA-DQ2.2 mediated celiac disease. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 3063–3073. [CrossRef] [PubMed]
32. Petersen, J.; Kooy-Winkelaar, Y.; Loh, K.L.; Tran, M.; Van Bergen, J.; Koning, F.; Rossjohn, J.; Reid, H.H. Diverse T Cell Receptor Gene Usage in HLA-DQ8-Associated Celiac Disease Converges into a Consensus Binding Solution. *Structure* **2016**, *24*, 1643–1657. [CrossRef] [PubMed]
33. Petersen, J.; Van Bergen, J.; Loh, K.L.; Kooy-Winkelaar, Y.; Beringer, D.X.; Thompson, A.; Bakker, S.F.; Mulder, C.J.J.; Ladell, K.; McLaren, J.E.; et al. Determinants of Gliadin-Specific T Cell Selection in Celiac Disease. *J. Immunol.* **2015**, *194*, 6112–6122. [CrossRef] [PubMed]
34. Henderson, K.N.; Tye-Din, J.A.; Reid, H.H.; Chen, Z.; Borg, N.A.; Beissbarth, T.; Tatham, A.; Mannering, S.I.; Purcell, A.W.; Dudek, N.L.; et al. A Structural and Immunological Basis for the Role of Human Leukocyte Antigen DQ8 in Celiac Disease. *Immunity* **2007**, *27*, 23–34. [CrossRef] [PubMed]
35. Tollefsen, S.; Hotta, K.; Chen, X.; Simonsen, B.; Swaminathan, K.; Mathews, I.I.; Sollid, L.M.; Kim, C.-Y. Structural and Functional Studies oftrans-Encoded HLA-DQ2.3 (DQA1*03:01/DQB1*02:01) Protein Molecule. *J. Biol. Chem.* **2012**, *287*, 13611–13619. [CrossRef] [PubMed]
36. D'Auria, G.; Vacatello, M.; Falcigno, L.; Paduano, L.; Mangiapia, G.; Calvanese, L.; Gambaretto, R.; Dettin, M.; Paolillo, L. Self-assembling properties of ionic-complementary peptides. *J. Pept. Sci.* **2008**, *15*, 210–219. [CrossRef]
37. Parrot, I.; Huang, P.C.; Khosla, C. Circular Dichroism and Nuclear Magnetic Resonance Spectroscopic Analysis of Immunogenic Gluten Peptides and Their Analogs. *J. Biol. Chem.* **2002**, *277*, 45572–45578. [CrossRef]
38. Güntert, P. Automated NMR Structure Calculation with CYANA. *Protein NMR Tech.* **2004**, *278*, 353–378. [CrossRef]
39. Koradi, R.; Billeter, M.; Wüthrich, K. MOLMOL: A program for display and analysis of macromolecular structures. *J. Mol. Graph.* **1996**, *14*, 51–55. [CrossRef]

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Review

Pro-Pre and Postbiotic in Celiac Disease

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Abstract: Celiac Disease (CD) is an autoimmune disease characterized by inflammation of the intestinal mucosa due to an immune response to wheat gliadins. It presents in subjects with genetic susceptibility (HLA-DQ2/DQ8 positivity and non-HLA genes) and under the influence of environmental triggers, such as viral infections and intestinal microbiota dysbiosis. The only treatment currently available in CD is a gluten-free diet for life. Despite this, the intestinal dysbiosis that is recorded in celiac subjects persists, even with adherence to dietary therapy. In this review, we have analyzed the literature over the past several decades, which have focused on the use of pro-, pre- and post-biotics in vitro and in vivo in CD. The study of probiotics and their products in CD could be interesting for observing their various effects on several different pathways, including anti-inflammatory properties.

Keywords: celiac disease; pro- pre- and post-biotic; inflammation

Citation: Conte, M.; Porpora, M.; Nigro, F.; Nigro, R.; Budelli, A.L.; Barone, M.V.; Nanayakkara, M. Pro-Pre and Postbiotic in Celiac Disease. *Appl. Sci.* **2021**, *11*, 8185. https://doi.org/10.3390/app11178185

Academic Editor: Giovanna Traina

Received: 23 July 2021
Accepted: 31 August 2021
Published: 3 September 2021

1. Introduction

Celiac disease (CD) is a common systemic disease that primarily affects the small intestine [1], due to the abnormal response of the immune system to gluten ingestion. It happens in subjects with genetic susceptibility (HLA-DQ2/DQ8 positivity and non-HLA genes) and under the influence of environmental triggers, including, a part from gluten, viral infections and intestinal microbiota dysbiosis [2]. Although 30–40% of the world population carry the HLA DQ2/DQ8 genotype, only 1–1.5% of them express the CD phenotype, which means that other genes together with other environmental factors may take part [2,3]. Typically, the inflammation in CD includes an increased intraepithelial lymphocyte (IEL) count, most often >25/100 cells [4,5]. Other features of CD are the presence of an adaptive T-cell-mediated response to gluten [6,7] and of specific endomysial antibodies (EMA, also called AEA), anti-tissue transglutaminase antibodies (TTG, a-tTG, TTA), and/or deamidated antigliadin antibodies (DGP) that play an important role in the serological work-up for CD. These antibodies strongly support the diagnosis of CD. In children, the intestinal biopsy is necessary where the antibody title is low and symptoms are absent. In paediatric patients with high tTG-IgA, ESPGHAN recommends that the decision whether or not to perform duodenal biopsies should be made during a shared decision making process between the paediatric gastroenterologist/coeliac disease specialist, the parent(s)/carer(s), and if appropriate, the child and any way when patients are on a gluten-containing diet [8]. The diagnosis is confirmed by the finding of an intestinal athrophy with shortening of the intestinal villi and increase of the crypt proliferation. Gluten is

poorly digested in the human intestine with or without CD. Undigested gluten peptides cross intact the mucosa of the small intestine. In the submucosa of the small intestine the human enzyme transglutaminase 2 (tTG) deamidates gluten peptides, leading to an increase in the affinity binding to human leucocyte antigen (HLA) DQ2 and HLA DQ8 molecules, subsequently triggering an inflammatory reaction in patients with CD [7]. The only treatment currently available in CD is a gluten-free diet for life. Recently, in CD, a role for the gut microbiota, has been described. There are several cross-sectional studies comparing microbiota in patients and controls, which describe a dysbiosis in CD patients before, and after, the gluten free diet. The aim of this review is to describe the most recent literature on pro-, pre and post-biotic in CD (See Figure 1).

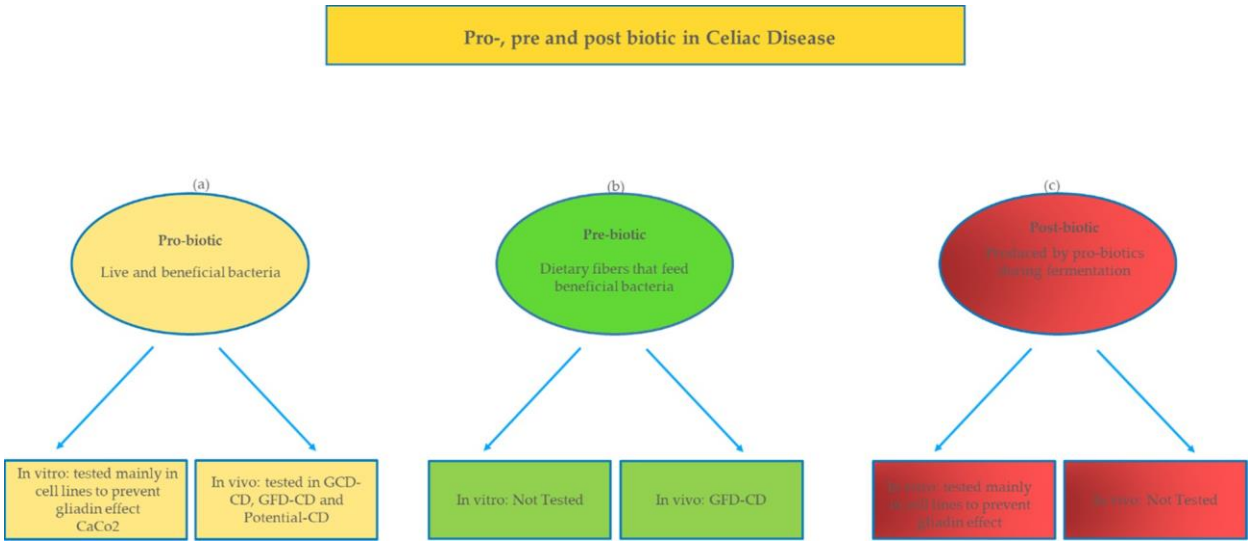

Figure 1. Schematic representation of the in vitro and in vivo experiments performed in CD with pro (**a**), pre (**b**) and post-biotics (**c**).

Pro-biotics are microorganisms, most of which are bacteria similar and/or the same to the beneficial bacteria that occur naturally in the human gut. Pro-biotics have been widely studied in a variety of gastrointestinal diseases. Pro-biotics have an important role in the maintenance of immunologic equilibrium in the gastrointestinal tract through the direct interaction with immune cells. Pro-biotic effectiveness can be species-, dose- and disease-specific, and the duration of therapy depends on the clinical indication.

Dietary fibers can be metabolized by microbes in the gastrointestinal tract. Human food enzymes are unable to digest most complex carbohydrates and plant polysaccharides. Instead, these polysaccharides are metabolized by microbes that generate short-chain fatty acids (SCFAs), including acetate, propionate and butyrate.

Post-biotics are known as “non-viable probiotics”, “inactivate probiotics” or “ghost probiotics” and refer to both non-viable microbial cells and soluble factors secreted by live bacteria or released after their lysis, including various cell surface components, lactic acid, short-chain fatty acids (SCFAs) and bioactive peptides among other metabolites. When administrated in sufficient amounts, these may contribute to the improvement of host health, even though the exact mechanisms are not yet well-known. The bacterial inactivation usually occurs by a mild heat treatment. The advantages of using post-biotics, include their higher stability, as they do not contain living bacteria, and; their higher levels of safety compared to pro-biotics, as they reduce the risk of microbial translocation, infection or enhanced inflammatory responses in consumers with imbalanced or compromised immune systems.

2. Materials and Methods

Pub Med search of articles on "Celiac Disease and Pro-biotics", Celiac Disease and Pre-biotics", "Celiac Disease and Post-Biotics" have been done from these we have selected literature with 6/68 references from the last 20 years, 14/68 from the last 15 years, 18/68 references from the last 10 years and 30/68 from the last 5 years

3. Microbiota Is Altered in CD

The microbiota is the ecological community of microorganisms within a defined environment. It is concentrated in the intestinal tract and is rapidly altered by external factors. The microbiome is the collective genomes of all microorganisms from a given environmental niche. Changes in the microbiota and consequently in the microbiome, impact the homeostasis of the whole body. The composition of the gut microbiota depends on many factors, such as age, geographical position, diet, genetics, natural birth and breast-feeding, so it is impossible to have the same microbiota in everybody and consequently to define the ideal microbiota. The gut microbiota influences immunity system response and inflammation. The microbiota plays a fundamental role in induction, training, and functioning of the host immune system so its alteration is generally associated with the immunity system response and inflammation [9]. In CD, alterations of the microbiota have been found. Interestingly the microbiota of CD patients can change in the different stage of the disease: There are microbiota signatures different for GCD-CD (Gluten Containing Diet Celiac Disease) and GFD-CD (Gluten Free Diet-Celiac Disease) respect to non CD subjects.

3.1. Changes in the GCD-CD Microbiota Respect to Controls

Patients in the acute phase of the disease at GCD present alteration of the gut microbiomawith increasing *E. coli*, ML615J-28, *Slackia*, *Victivallaceae*, *Enterobacteriaceae*, *Clostridiaceae*, *Coriobacteriaceae* and unclassified specie of *Clostridiales* and *Lachnospriraceae*, decreasing *C. lituseburense*, *Lactobacillus*, *F. Prausnitzii*, *Bifidobacterium*, *Dorea*, *B. wexlerae*, *Lachnospriraceae*, *A. hadrus*, *E. hallii*, *Veillonellaceae*, *R. bromi*, *R. faecis* CD patients showed a gut dysbiosis [10]. In these patients the bacteria that have a protective effect such as *Bifidobacteria*, *Firmicutes*, *Lactobacilli* and *Streptococci* are lower compared to healthy controls. Active CD patients have an increased of gram-negative bacteria like *Bacteriodes*, *Bacterioidetesfragilis, Prevotella*, *E. coli*, *Proteobacteria*, *Haemophilus*, *Serratia*, *Klesbisella* [11,12]. Patients with active CD showed a strong presence of *Proteobacteria* phylum and *Neisseria flavescence*, *Firmicutes* and *Actinobacteria* are less abundant [13]. In the gut microbiota of CD patients, the presence of pathogenic bacteria like *Clostridium perfringens* and *C. difficile* may be a consequence of the *Bifidiobacteria* reduction and apparently seem to promote the risk of developing celiac disease in patient at risk [14]. Moreover, a reduction of *Bifidobacterium* and *Lactobacillus* population is present in these patients, that may be protective against enteric infections like infection due to *C. difficile*. Patients with high risk of CD showed a decreased of *Bacteriodes*, *Prevoltella* and *Bifobacteria*.

3.2. Changes in the GFD-CD Microbiota Respect to GCD-CD

CD patients at GFD for at least 2 years showed a faecal microbiota composition similar to healthy controls characterized by a reduced diversity of *Lactobacillus* and *Bifidobacterium* species indicating that GFD could normalize gut microbiota composition in CD [15]. Untreated celiac patients showed a lower concentrations of *Lactobacilli* and a significant higher concentration of *Clostridium* vs. healthy volunteers and CD relatives. The data are dependent on the time of the GFD suggesting that a prolonged GFD could modify SCFA proteolytic patterns. Dysbiosis with its imbalance of commensal microbiota composition could influence the metabolism of gluten proteins in CD patients [16,17].

3.3. Changes of the Microbiota of Healthy Subjects at GFD

The GFD in the healthy subjects influence the gut microbiota reducing the *Bifidobacterium*, *Clostridium lituseburense e Faecali bacterium praunsnitzii* and increasing the *Enterobac-*

teriaceae and *Escherichia coli* [18]. One study showed that the bacterial profile remained relatively stable in healthy individuals on GFD but during the GFD period decreased *Veillonellaceae*, *Ruminococcusbromii* and *Roseburiafaecis*. Whereas, *Victivallaceae*, *Clostridiaceae*, ML615J-28, *Slackia* and *Coriobacteriaceae* increased were described [19]. Moreover, using a low gluten diet, it is possible to observe an increase in unclassified species of *Clostridiales* and *Lachnospiraceae*. While, *E. hallii* and *A. hadrus*, *Dorea* and *T. blautia*, two species of the *Lachnospiraceae* and four species of *Bifidobacterium* decreased [20]. The GFD influences the gut microbiomain non-CD subjects increasing *E. Coli*, ML615J-28, *Slackia*, *Victivallaceae*, *Enterobacteriaceae*, *Clostridiaceae, Coriobacteriaceae* and unclassified specie of *Clostridiales* and *Lachnospriraceae*, decreasing *C. lituseburense, Lactobacillus*, *F. prausnitzii*, *Bifidobacterium, Dorea, B. wexlerae, Lachnospriraceae, A. hadrus, E. hallii, Veillonellaceae, R. bromi, R. faecis.*

In conclusion, CD patients present a different microbiome respect to normal subjects in the acute phase of the disease, while eating gluten. GFD can modulate gut microbiota of CD patients respect to healthy subjects, and can induce some alterations in the microbiota in non-CD subjects at GFD. Most studies on gut microbiota in CD are descriptive, including different categories of patients on GFD, GCD, at risk both with, and without, symptoms. From these studies it is difficult to determine whether the onset of CD is due to the alteration of microbiota or whether this is secondary to the intestinal damage present in CD.

In this context, pre-probiotics could have the characteristics to be useful to prevent or even ameliorate dysbiosis in CD. Post-biotics have not been tested in CD yet, but from the in vitro data they seem to be able to prevent some gliadin and gliadin peptides effects.

4. In Vitro Assays to Study Pro-Pre and Post Biotic in CD

Many pro-, pre- and post-biotics have been tested in intestinal epithelial cells in culture for their ability to prevent gliadin effects. The most used cell line is CaCo-2 an intestinal epithelial cell line derived from a human colorectal adenocarcinoma. Although, these cells transformed, they have the ability to differentiate in vitro toward intestinal epithelial cells. Once differentiated they produce tight junctions and absorptive-secretive ability.

The read outs used to study pro-, pre- and post-biotics in vitro were various. Generally, integrity of the epithelial layer, inflammation and innate immunity were analyzed. Following is an analytical division of pro-pre-post-biotics used and the main effects analyzedin vitro. The main read outs were tested before and after gliadin or gliadin peptides treatment. Following is a list of the main pro, pre and post-biotic used on this cellular model.

Principal read-outs used to study probiotics effect on CaCo-2 cells before and after gliadin or gliadin peptides treatment.

a. Permeability (TEER)
b. Toxicity experiments
c. Analysis of pro-inflammatory markers
d. Intestinal organoids

4.1. Permeability of Intestinal Epithelial Cells

Celiac disease is characterized by enhanced intestinal paracellular permeability probably due to alterations of tight junction (TJ) proteins function and expression. Intestinal permeability in CD and abnormal handling of gluten peptides by epithelial cells might activate the local immune system excessively and induce the disease [21]. TEER, one of the most popular methods to study permeability in epithelial cells is a non-invasive technique that consist of a measurement in electrical resistance across a cellular monolayer. It is regarded as a very sensitive and reliable method for confirming the integrity and permeability of the monolayer. The CaCo-2 monolayer generates a TEER of 150–400 Ω/cm^2 any insult that alter the monolayer will lower it. For these reasons, the CaCo-2 cells are often used to study nutrients and drugs transport. They also allow to study the main routes of transport [22].

4.2. Toxicity and Analysis of Pro-Inflammatory Markers

Gliadin peptides with specific amino acid sequences have proven to trigger pro-inflammatory cell responses. These involve activation of the nuclear factor kappa-B (NF-kB) in small intestinal mucosa of celiac patients [23], and increased expression of proinflammatory cytokines related to the innate immune response, such as tumor necrosis factor a (TNF-a) [24] and interleukine (IL) 1b [25].

5. Probiotics

5.1. Bifidobacteria

Bifidobacteria are Gram-positive microorganisms with a high G + C DNA content. In 1899 they were isolated from the faeces of a breastfed infant. They are strictly anaerobic and occur in uniform Y- and V-shaped branched and bifurcated, spatulate or club-shaped forms; they are not motile and do not form spores. The branched appearance of *bifidobacteria* depends not only on the strain but also on the medium used for cultivation. The genus *Bifidobacterium* consists of 48 different taxa, of which 40 have been isolated in the gastrointestinal tract (GIT) of mammals, birds or insects, the remaining eight from sewage and fermented milk [26–28]. Acetic and lactic acids are produced by glucose fermentation in a ratio of 3:2 [27]. There are 32 species of *bifidobacteria* (Table 1) identified by fermentation assays, observations of cell morphology, and electrophoretic mobility of enzymes such as transaldolase (14 types) or 6-phosphogluconate dehydrogenase (19 types; 32) [29].

Table 1. *Bifidobacterium* Species.

B. adolescentis *	*B. dentium* *	*B. minimum*
B. angulatum *	*B. gallicum* *	*B. pseudocatenulatum* *
B. animalis	*B.gallinarum* *	*B. pseudolongumsubsp. Globosum*
B. asteroides	*B. globosum*	*B. pseudolongumsubsp. Pseudolongum*
B. bifidum	*B. indicum*	*B. pullorum*
B. boum	*B. infantis* *	*B. ruminantium*
B. breve *	*B. inopinatum*	*B. saeculare*
B. catenulatum *	*B. lactis*	*B. subtile*
B. choerinum	*B. longum* *	*B. suis*
B. coryneforme	*B. magnum*	*B. thermophilum*
B. cuniculi	*B. merycicum*	

* Detected in human faeces.

Laparra and Sanz Y. (2010) demonstrated on CaCo-2 cells, the effects of *Bifidobacterium* strains (*B. IATA-ES2, B. longum IATA-ES1*, and *B. animalis IATA-A2*) by analyzing the peptide sequences produced by gastrointestinal digestion of gliadins and comparing toxic and proinflammatory effects.

By RP-HPLC-ESI-MS/MS different patterns of gliadin peptides were detected in samples inoculated with *bifidobacteria* compared to those not inoculated. Most of the peptides generated in the samples inoculated with *bifidobacteria* showed lower molecular mass than those generated in the non-inoculated samples (2500 Da) during the in vitro digestion [30].

Gliadin peptides resulting from in vitro digestion, inoculated or not, with *B. animalis* and *B. bifidum* were cytotoxic to intestinal epithelial cells in vitro. Whereas, those inoculated with *B. longum* were not. Furthermore, the analysis of pro-inflammatory cytokines showed that NF-kB, IL-1β TNF-a ($p < 0.05$) were consistently reduced when digested gliadin was inoculated with all bifidobacterial strains compared with non-inoculated digested gliadin [30].

Olivares M. et al. (2012) demonstrated by MALDI-TOPF techniques that digested gliadin up-regulates proteins involved in actin rearrangement, inflammation and apoptosis in CaCo-2, while *B. longum* CECT 7347 was able to reduce them (21 vs. 9). Many of these proteins were also involved in calcium homeostasis, cell survival and function [31].

de Almeida N et al. (2020) evaluated the effect of *Bifidobacterium* species: *B. bifidum, B. longum, B.breve, B. animalis* alone, and also a consortium of *Bifidobacterium* on the digestion of intact gluten proteins (gliadins and glutenins) and the associated immunomodulatory responses elicited by the resulting peptides. The readout was activation of NF-kB p65 and expression of the cytokines TNF-α and IL-1β in CaCo-2 cell cultures exposed to the peptides [32]. Giorgi A et al. (2020) used a multi-strain probiotic preparation containing two strains of *lactobacilli* (*L. paracasei, L. plantarum*) and three strains of *bifidobacteria* (two different *B. breve and B. animalis*). They evaluated the ability of a probiotic mixture to hydrolyze gluten peptides after simulated gastrointestinal digestion of gliadin (PT-gliadin). Protein and peptide mixtures, untreated or proteolyzed with the probiotic preparation, were analyzed before, and after, each proteolytic step by different techniques (SDS-PAGE, reverse-phase HPLC, filtration on different molecular shear membranes). PT-gliadin, untreated or digested with probiotics was then used to assess oxidative stress, IL-6 cytokine production, and expression of tight junction proteins, such as occludin and zonulin, in CaCo-2 cells. PT-gliadin induced IL-6 production and modulation and redistribution of zonulin and occludin, while digestion with probiotic strains reversed these effects [33] (See Table 2).

Table 2. In vitro study using *Bifidobacterium* and *Lactobacillus*.

Reference	Composition, Strains	Meaning
Laparra J.M. and Sanz Y. 2010 [30]	*Bifidobacterium strains (B. bifidum IATA-ES2, B. longum IATA-ES1, and B. animalis IATA-A2*	*Bifidobacterial* strains can inhibit the gliadin-induced cytotoxic and pro-inflammatory responses in intestinal epithelial cells
Olivares M et al., 2012 [31]	*Bifidobacterium longum CECT 7347*	*B. longum CECT 7347* reduces the toxic and inflammatory effects of gliadin-derived peptides
De Almeida and Natália Ellen Castilho et al. (2020) [32]	*Bacterium species B. bifidum BB-G90, B. longum BLG301, B. breve BB-G95and B. animalis L-G101*	*Bifidobacterium* species can able to assist the proteolysis of intact gluten proteins, gliadins, and glutenins, to form different peptide patterns, with reduction of cytotoxicity and proinflammatory response in intestinal epithelial cells
Giorgi A and et al., 2020 [33]	*Lactobacillus paracasei 101/37 LMG P-17504, Lactobacillus plantarum 14 D CECT 4528, Bifidobacterium animalis subsp. lactis Bi1 LMG P-17502, Bifidobacterium breve Bbr8 LMG P-17501 and Bifidobacterium breve BL10 LMG P-17500*	The probiotic strains tested canable to reduce the toxicity of gliadin

5.2. Lactobacillus

Lactobacillus, (genus Lactobacillus), are gram-positive, non-motile, ana- and aerobic, non-sporigenic bacteria. They are widely distributed in feed, silage, manure, milk, and dairy products. Despite continuous taxonomic changes in recent years, 70 species are recognized, and among them, 19 are of major interest in probiotic research (Table 3). From glucose metabolism, *lactobacillaceae* are able to produce lactic acid as a by-product, but also acetic acid, ethanol, carbon dioxide and other secondary compounds. For this reason, they are able to reduce pH and are used for the production of sour milk, cheese and yogurt, and play an important role in the production of fermented vegetables (pickles and sauerkraut), beverages (wine and juices), sourdough bread and some sausages. According to the type of Lactobacillus considered we have a different quantity of lactic acid produced: *L. acidophilus, L. casei and L. plantarum*, homofermentants produce 85% of it, primary by-product. In contrast, other species, such as *L. brevis and L. fermentum*, heteroferment glucose metabolism. *Lactobacilli* are commensal inhabitants of the animal and human gastrointestinal tracts, as well as the human mouth and vagina. Low levels of lactobacilli are present consistent findings in the microbiomes of adults and children with active CD [34].

Table 3. *Lactobacillus* Species Detected in the Intestinal Tract and/or used in Probiotic Products.

L. acidophilus	*L.delbrueckiisubsp. Bulgaricus*	*L. plantarum*
L. agilis	*L. gallinarum*	*L. rhamnosus*
L. aviarius	*L. gasseri*	*L. reuteri*
L. amylovorus	*L. johnsonii*	*L. murinus*
L. brevis	*L. hamsteri*	*L. ruminis*
L. casei	*L. intestinalis*	*L. salivarius*
L. crispatus		

Orlando A. et al. (2014/2018) have demonstrated that the administration of gliadin to CaCo-2 cells caused a significant alteration of paracellular permeabilityas by the rapid decrease in transepithelial resistance with a concomitant zonulin release causing also anincrease polyamine content. The co-administration of viable *Lactobacillus rhamnosus GG*, heat-killed *L.GG* (L.GG-HK) or its conditioned medium (*L.GG*-CM) preserves the intestinal epithelial barrier integrity. Viable *L.GG* and L.GG-HK, but not *L.GG*-CM, led to a significantreduction in the single and total polyamine levels showing that the presence of cellular polyamines is a pre-requisite for this probiotic to exert its capability in restoring paracellularpermeability by affecting the expression of different TJ proteins [35,36].

Sarno M. et al. (2014) have studied the effect of probiotic *Lactobacillus paracasei (LP) CBA L74* on the entrance of two peptides of gliadin: P31–43 and P57–68, the two main undigested peptides involved in CD pathogenesis, in CaCo-2 cells. Treatment of CaCo-2 cells with gliadin peptides, P31–43 and P57–68, conjugated with the fluorochrome lissamine (P31–43-liss and P57–68-liss) allowed to follow their entrance into the cells [37–39]. Pretreatment with *LP CBA L74* reduced the fluorescence intensity of both P31–43- and P57–68-liss inside CaCo-2 cells in a statistically significant way. Interestingly, *LP CBA L74* was more efficient in reducing P31–43-liss than P57–68-liss FI in CaCo-2 cells in a statistically significant way. This study describes a novel effect of probiotics [40].

6. Prebiotics

Pro-biotics are living microorganisms that mostly colonize the gastrointestinal (GI) tract. While, pre-biotics are fibres that cannot be digested or absorbed by the body. These act as a nutrient for probiotics, especially Bifidobacterium, increasing their numbers. They are defined as “non-absorbable food components that beneficially stimulate one or more groups of gut-friendly microbes and thus have a positive effect on human health” [41]. They can be metabolized by microbes in the gastrointestinal tract.

They positively affect the host by selectively stimulating the growth and/or activity of a limited number of bacteria. Naturally present in foods, such as artichokes, garlic, onions, and others. It may be necessary to consume large amounts of these foods to have a “bifidogenic” effect. For this reason, it is easiest to take a pre-biotic supplement or a combination of pro-biotic and pre-biotic (symbiotic) supplements to achieve optimal levels. They are, in addition, resistant to hydrolysis by digestive enzymes and are not absorbed in the upper part of the gastrointestinal tract. Upon reaching the large intestine, the site of the microbiota, they stimulate the growth of certain microorganisms [41]. Following their intestinal fermentation, SCFAs such as acetate, propionate and butyrate are produced [42].

Some of the most common pre-biotics are inulin and oligofructose. Oligosaccharides are non-digestible short-chain polysaccharides with numbers between 2 and 20 (approximately) saccharide units; hydrolysis of polysaccharides is a process that can be implemented to obtain different commercial products from dietary fiber, starch. An important pre-biotic is inulin, composed of a mixture of fructan chains and chicory roots are its main source of extraction. In vitro they stimulate the selective growth of Bifidobacterium [42].

In vitro, the use of prebiotics has not been studied, neither in CaCo-2 cells or in cells derived from CD patients. Many reports in the literature have been done in preventing the development of irritable bowel syndrome (IBS). For example, Qian Chen et al. (2017) explored the possible mechanisms using a prebiotic blend (PB) composed of fructo-

oligosaccharide (FOS), galacto-oligosaccharide (GOS), inulin and anthocyanins in CaCo-2 cells after co-incubation with PB and *Salmonella typhimurium* and in post-infectious IBS models in C57BL/6 mice. The results showed that PB significantly decreased pro-inflammatory cytokines in both infected CaCo-2 cells and PI-IBS models [43].

7. Post-Biotics

Post-biotics have been used in in vitro experiments in CaCo-2 cells daily to study their ability to prevent gliadin and gliadin peptides effects on CaCo-2 cells.

Sarno M. et al. have showed that *LP CBA L74* post-biotic was able to reduce gliadin peptides entrance in CaCo-2 cells [40].

Using 16S rRNA analysis of duodenal and oropharyngeal samples from CD patients and control subjects (Ctr), previously identified a peculiar *Neisseria flavescens* strain in adults affected by CD [13,44]. This bacterial strain, isolated from the above samples, induced an immune-inflammatory response in human and murine dendritic cells, in CaCo-2 cells, and in ex vivo duodenal mucosal explants of Ctr subjects, thereby suggesting that it could play a role in CD [13].

Labruna G et al. (2019) have evaluated if metabolism and trafficking was altered in CD-*N. flavescens*-infected CaCo-2 cells and if any alteration could be mitigated by pretreating cells with *LP CBA L74* supernatant, despite the presence of P31–43. CD-*N. flavescens* colocalised more than control *N. flavescens* with early endocytic vesicles and more escaped autophagy thereby surviving longer in infected cells. P31–43 increased colocalisation of *N. flavescens* with early vesicles. Mitochondrial respiration was lower in CD-*N. flavescens*-infected cells versus not-treated CaCo-2 cells, whereas pretreatment with *LP CBA L74* reduced CD-*N. flavescens* viability and improved cell bioenergetics and trafficking. CD-*N. flavescens* induces metabolic imbalance in CaCo-2 cells [45].

Sarno M. et al. have observed that *LP CBA L74* was able to ferment several different cereals. Fermented oats, rice and wheat were used to test the effect of *LP CBA L74* on P31–43 entrance after fermentation. P31–43-liss entrance was reduced after treatment with fermented oat (FI reduction 56%), rice (FI reduction 35%) and wheat (FI reduction 24%). These data suggest that *LP CBA L74* effect on P31–43-liss entrance was still present after fermentation of different cereals and that could be linked to compounds produced during the fermentation process and not to alive bacteria [40].

Gallo M. et al. have investigated if pH control during fermentation process of rice with probiotic *LP CBA L74*, could improve the bacterial growth and the lactic acid production and also the ability to interfere P31–43 entrance in the CaCo-2 cells. During rice fermentation process pH control greatly improved the lactic acid production. Both rice fermented with and without pH control, were able to prevent P31–43-liss entrance even after they were washed away from the cells, triggering into the cells a sort of memory that could prevent the entrance of P31–43-liss for several hours. This information could be useful in the field of nutrition; it suggests that the effect of certain post-biotics could last also after they have left the intestine [46] (See Table 4).

Recently, Freire R et al. using intestinal organoids developed from duodenal biopsies from both non-celiac (NC) and celiac (CD) patients, they analyzed the role of microbiota-derived molecules in modulating the epithelium's response to gluten. Therefore, the authors selected three bacterial bioproducts: Butyrate, lactate, and PSA derived from *Bacteroides fragilis*.

The authors analyzed the expression of genes related to gut barrier function, found altered in CD. Butyrate significantly upregulated the expression of MUC5AC, MUC6, TFF1 and CLDN18, in CD organoids. PSA significantly increased the expression of CLDN18 only, while lactate did not alter the expression of the analyzed gut barrier function-associated genes. Similar regulation of genes was observed in NC organoids.

Table 4. In vitro study using *LP CBA L74* post-biotc.

Reference	Composition, Strains	Meaning
Sarno M et al., 2014 [40]	*Lactobacillus Paracasei CBA L74*	The postbiotic *L. paracasei-CBA L74* interferes with gliadin peptides entrance in epithelial cells
Labruna G et al. (2018) [45]	*Lactobacillus Paracasei CBA L74*	*N. flavescens* strain induces imbalance in the mitochondrial respiration of CaCo-2 epithelial cells and was able to altered the trafficking pathway. This metabolic alteration appears to be in part reversed by the post-biotic *L. paracasei-CBA L74*, irrespective of the presence of the P31-43 peptide
Gallo M et al., 2015 [46]	*Lactobacillus Paracasei CBA L74*	During rice fermentation process pH control greatly improved the lactic acid production and also the ability to interfere P31-43 entrance in the CaCo-2 cells.

Moreover, they have analyzed the effect of the bacterial bioproducts on cytokines released by the CD monolayers challenged with gliadin, all bioproducts exerted a global protective effect by reducing the pro-inflammatory cytokine secretion triggered by PTG. However, only lactate and butyrate significantly reduced the secretion of IL15 and IFNγ cytokines, respectively in CD [47].

8. Clinical Trials Using Pro- and Pre-Biotic in CD

8.1. Probiotics and Celiac Disease

The intestinal microbiota has a very close relationship with the individual; provides a barrier against pathogen colonization, synthesize vitamins and other beneficial compounds, and stimulate the immune system. Celiac disease has been associated with a condition of dysbiosis, but no study has ever shown that there is a characteristic "celiac intestinal microbiota" [48]. The intestinal microbiota plays a fundamental role in maintaining and improving intestinal health and the whole organism [49]. Dysbiosis is present in the celiac patient, but it is still not clear whether this is the cause or consequence of the disease, it must be said that this dysbiosis persists regardless of adherence to a GFD and is partly related to this particular diet. Indeed, GFD influences the composition of the intestinal microbiota due to a reduction in the intake of polysaccharides [50]. Probiotics could help restore celiac dysbiosis. In this review, we analyzed various intervention studies conducted in the last 10 years (2011–2021), which envisaged the use of pro-biotics as an intervention. In particular, the analyzed works can be divided into two broad categories: Intervention studies, which evaluated whether the introduction of a pro-biotic could improve some parameters of subjects with CD in association with a GFD, and; studies which evaluated whether the administration of pro-biotics could influence the clinical course of the disease both in patients with untreated CD and in potential subjects (See Table 5).

Table 5. Effect of probiotics in vivo studying on GFD CD.

Reference	Intervation	Read Out	Effects
Olivares M et al. British Journal of Nutrition 2014 [51]	*B. longum CECT 7347* for 3 months in in thirty-three children	Evaluate the effects of *B. longum CECT 7347* administration on immune and anthropometric parameters, and on intestinal microbiota composition.	Reductions of pro-inflammatory bacteria (*B. fragilis group*), activated T lymphocytes and inflammatory markers (TNF-α)

Table 5. *Cont.*

Reference	Intervation	Read Out	Effects
Klemenak, M et al. *Dig Dis Sci* 2015 [52]	*Bifidobacterium breve BR03 and B. breve B632* for 3 months in 49 children	Investigate the effect of two probiotic strains on serum production of anti-inflammatory cytokine interleukin 10 (IL-10) and pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α)	Low production of TNF-α
Quagliariello et al. Nutrients 2016 [53]	*B. breve BR03 (DSM 16604) and B. breve B632 (DSM 24706)* for 3 months in 40 children	Impact of the administration of two *Bifidobacterium breve* strains on the gut microbiota composition	An increase of *Actinobacteria* and a re-establishment of the physiological *Firmicutes/ Bacteroidetes* ratio
Harnett J et al. Evidence-Based Complementary and Alternative Medicine 2016 [54]	*VSL*#3 for 12 weeks 45 patients	Effects of a probiotic supplement on the CD microbiota	No effects
Francavilla R et al. J ClinGastroenterol. 2019. [55]	Mixture of 5 strains of lactic acid bacteria and *bifidobacteria* * for six months in 109 patients	Evaluate the efficacy and safety of probiotic mixture in CD patients with IBS-type symptoms despite a strict GFD.	Improving the severity of IBS-type symptoms, modification of gut microbiota, by an increase of *bifidobacteria.*

* *Lactobacillus casei* LMG 101/37 P-17504, *L. plantarum* CECT 4528, *Bifidobacterium animalis* subsp. *lactis* Bi1 LMG P-17502, *B.breve* Bbr8 LMG P-17501, *B. breve* Bl10 LMG P-17500.

8.1.1. Administration of Pro-Biotics in Patients with CD at GFD with or without Gluten Challenge

(a) Effect of pro-biotics associated with a gluten-free diet

In a study by Olivares M et al. [51] *B. longum CECT 7347* was given for three months to children with CD in combination with a GFD diet. The intervention resulted in; an increase in the height percentile compared to placebo treatment; significant reductions in mature T lymphocytes (CD3þ), and; HLA-DRþ T lymphocytes and reductions in TNF-a compared to placebo treatment. Moreover, in the study by Klemenak M et al. [52], the daily administration for three months of *B. short BR03* and *B632* in association with the GFD diet in subjects with CD led to a significant decrease in serum TNFα levels compared to the placebo group. In the study by Quagliariello A et al. [53] the administration of a mixture of 2 strains, *B. short BR03* (DSM 16604) and *B. short B632* (DSM 24706) (1:1) for three months in subjects with CD in association with GFD induced the restoration of the physiological *Firmicutes/Bacteroidetes* relationship in the intestinal microbiota, and led to an increase in members of the *Actinobacteria* phylum but this increase was not significant. In a multicenter study, the probiotic formulation VSL # 3 was administered for 12 weeks in 45 subjects with CD who reported only partial improvement in symptoms despite adhering to a strict GFD [54]. While, in the study of Francavilla R et al. [55], the 6-week intervention of a mixture of 5 strains of lactic acid bacteria and bifidobacteria: *Lactobacillus casei* LMG 101/37 P-17504, *Lactobacillus plantarum* CECT 4528, *Bifi-dobacterium animalis subsp. lactis* Bi1 LMG P-17502, *Bifidobacterium short* Bbr8 LMG P-17501, *B. short* Bl10 LMG P-17500, in patients suffering from CD at GFD with symptoms, led to a decrease in pain perception and a modification of the intestinal microbiota, characterized by an increase in *bifidobacteria* which is still detectable 6 weeks after stopping probiotics.

(b) Effect of pro-biotics on the gluten challenge in CD

In the study by Edgardo Smecuol et al. [56] the administration, in subjects with CD, for 3 weeks and of *Bifidobacterium infantis natren* life in conjunction with the daily con-sumption of 12 g of gluten led to a significant improvement in gastrointestinal symptoms compared to the placebo group. It is conceivable that the observed beneficial effect may be related to

the modulation of innate immunity. In this regard, a second study was conducted in which the administration *of B. infantis natren* Life Start super strain in sub-jects with CD at GFD with challenge of 12 g of gluten resulted in reductions in α-defensin and in the number of Paneth cells in duodenal biopsy samples [57].

8.1.2. Effect of Pro-Biotics in High-Risk Individuals

Subjects with positive genetics and first degree familiarity for celiac disease are regarded as "at risk".

The European multicentre Prevent Celiac Disease project investigated the possible primary prevention of celiac disease [58]. What they wanted to test was whether the frequency of celiac disease could be reduced by exposing children at high risk of the disease to small amounts of gluten between 16 and 24 weeks of age, preferably while still breastfeeding. The intervention consisted of randomly administering participants 200 mg of gluten mixed with 1.8 g of lactose daily for 8 weeks. The treatment did not reduce the risk of disease. In second study 832 infants were randomly assigned to two groups: Group A: gluten introduction at 6 months of age; Group B: gluten introduction at 12 months of age. Delayed gluten introduction did not change the risk of celiac disease among at-risk children, but delayed gluten introduction was associated with a delayed onset of the disease [59]. The Environmental Determinants of Diabetes in the Young (TEDDY) study evaluated whether the intake of *L. reuteri* and *L. rhamnosus*, during the first year of life, could influence the development of CD. The treatment had no protective effect [60].

8.1.3. Effect of Pro-Biotics in Potential Subjects

Potential Celiac Disease (PC) is characterized by the detection of specific antibodies in the serum, the presence of compatible HLA, but in the absence of alterations of the intestinal mucosa (Type 0, 1 according to Marsh). Patients with PC may or may not have clinical symptoms [8].

In the intervention study by Hakansson A et al., administration of *L. plantarum* and *L. paracasei* for 6 months in potential subjects on a normal containing gluten diet had modulatory effects on the peripheral immune response; but it showed no effect in preventing the progression of the disease, which developed in the same proportion in both groups [61].

9. Pre-Biotics as Food Supplements in GFD

The gluten-free diet exposes celiacs to nutritional deficiencies. In fact, the consumption of some nutrients, in particular fiber, iron, calcium and folic acid, is lower than normal in patients who follow a gluten-free diet. One possible strategy for avoiding the nutritional deficiencies in CD patients is appropriate dietary supplementation. According to the most recent definition, proposed by the International Scientific Association of Pro-biotics and Pre-biotics in 2017, pre-biotics are substrates that are used by the intestinal flora as an energy source; these substrates have a beneficial effect on human health [62]. Prebiotics can have beneficial effects on the absorption of vitamins and minerals; some of them have been shown to improve mineral bioavailability and increase iron absorption in animal studies [63].

To date, all interventions with pre-biotics have been tested in celiac subjects at GFD with the aim of improving the subjects' nutritional status.

In a randomized clinical trial with a placebo conducted on 34 patients with celiac disease, diagnosed according to the criteria created by the European Society of Gastroenterology, Hepatology and Pediatric Nutrition (ESPGHAN criteria 2012) the administration of 10 g per day of Sinergy 1 (oligofructose-enriched inulin, ORAFTI, Tienen, Belgium) resulted in a significant reduction of hepcidin (regulator of iron metabolism in the intervention group). No differences were found in the morphological and biochemical parameters of the blood (for details see Table 6) [64]. Natalia Drabińska et al. observed that patients with GFD may counteract the reduction in *Bifidobacterium* count in CD children after the administration of Synergy 1. The addition of Synergy 1 modified the faecal SCFA profile,

and in particular, increased the concentration of acetates. In a second intervention study, the levels of 25-hydroxyvitamin D [25 (OH) D], parathyroid hormone, vitamins E and A, calcium, phosphate, magnesium, total protein and albumin were evaluated after administration of 10 g a day of Sinergy 1 for three months. The Synergy 1 leds to an increase in 25 (OH) D and vitamin E, but there was no difference in the levels of parathyroid hormone, calcium, phosphate and vitamin A [65,66]. The circulating amino acid (AAs) concentrations are indicators of dietary protein intake and metabolic status. In CD, the AA imbalance is frequently observed. In two other papers, the integration of Sinergy 1 for three months led to an increase in urinary excretion of AA with a simultaneous increase in the content of circulating AAs, which could be attributed to higher absorption and/or intensified metabolism of AAs, as well as further healing of the intestinal mucosa [67]. Changes in Glu concentration suggest that oligofructose-enriched inulin could improve the intestinal condition and permeability, leading to benefits in bone metabolism, increasing bone formation rates and reducing bone resorption rates [68].

Table 6. Effect of prebiotics in vivo study.

Reference	Read Out	Effects
Feruś, K et al. Nutrients 2018 [63]	Evalutate the effect of oligofructase-enriched inulin (Synergy 1) on iron homeostasis in CD children GFD	Reduction in serum hepcidin concentrations, no change in blood morphological and biochemical parameters (including ferritin, hemoglobin and C-reactive protein (CRP)
Drabińska, N et al. Nutrients 2018 [65]	Evaluate the effect of prolonged oligofructose-enriched inulin (Synergy 1) administration on the characteristics and metabolism of intestinal microbiota in CD children GFD	Increase *Bifidobacterium* and modified the faecal SCFA profile, increasing the concentration of acetate
Drabińska, N Amino Acids 2018 [67]/Nutrients 2018 [66]	Analyze the effect of the intervention on plasma and urinry concentrations of AA	Synergy 1 could improve the intestinal condition and permeability
Drabińska, N et al. Nutrients 2018 [67]	Determine the variations of 25-hydroxyvitamin D[25(OH)D], parathyroidhormons, vitamines E and A, calcium, phosphate, magnesium, total protein and albumin	Improved vitamin D and vitamin E status in children and adolescents with CD
Drabińska, N et al. Bone 2019 [68]	Effect of oligofructase-enriched inulin (Synergy 1) on bone turnover markers and immune response in children in CD children GFD	Improved bone metabolism increased bone formation rates and decreased bone resorption process rates. The intervention did not lead to immunological response changes

Type of study: Randomized double blind. Sample size: 34 children. Follow up: 3 months. Inclusion criteria: ESPGHAN 2012. Intervation: 10 g of oligofructose-enriched inulin (Synergy 1) or a placebo (maltodextrin).

10. Conclusions

The few reports of in vivo trials with pro-biotics in CD indicate that they are not able to prevent the disease. Pro-biotics only seem to be effective in alleviating symptoms. These conclusions are in line with other reports on Crohn's Disease and Ulcerative Colitis. Pre-biotics alone do not seem to be very active in vivo, whereas post-biotics have never been used in vivo.

In vitro post-biotics in intestinal epithelial cells seem to be very effective in preventing gliadin and gliadin peptides effects. Post-biotics seem to be effective in many different pathways that ultimately point to the prevention of the inflammation induced by gluten. The use of in vitro patient-derived organoids to model CD pathogenesis could be a novel tool to further study CD treatment and prevention

It is not clear what aspect of the post-biotic induces this activity, although some reports point to small protein/s produced by the bacteria. Taken all together, these data point to a

possible application of post-biotics in the prevention of inflammation in CD patients at risk of the diseases. Only in vivo trials will be able to confirm this application.

Author Contributions: M.V.B., M.C., M.P. and M.N.: have equally contributed to conceptualization, methodology, investigation, resources, data curation, writing. F.N., A.L.B., R.N. have equally contributed to methodology, resources, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rubio-Tapia, A.; Murray, J.A. Updated guidelines by the European Society for the Study of Coeliac Disease. *United Eur. Gastroenterol. J.* **2019**, *27*, 581–582. [CrossRef]
2. Caio, G.; Volta, U.; Sapone, A.; Leffler, D.A.; de Giorgio, R.; Catassi, C.; Fasano, A. Celiac disease: A comprehensive current review. *BMC Med.* **2019**, *17*, 1–20. [CrossRef]
3. Caio, G.; Lungaro, L.; Segata, N.; Guarino, M.; Zoli, G.; Volta, U.; De Giorgio, R. Effect of gluten-free diet on gut microbiota composition in patients with celiac disease and non-celiac gluten/wheat sensitivity. *Nutrients* **2020**, *19*, 1832. [CrossRef]
4. Walker, M.M.; Murray, J.A.; Ronkainen, J.; Aro, P.; Storskrubb, T.; D'Amato, M.; Lahr, B.; Talley, N.J.; Agreus, L. Detection of Celiac disease and Lymphocytic Enteropathy by parallel serology and histopathology in a population-based study. *Gastroenterology* **2010**, *139*, 112–119. [CrossRef]
5. Corazza, G.R.; Villanacci, V.; Zambelli, C.; Milione, M.; Luinetti, O.; Vindigni, C.; Chioda, C.; Albarello, L.; Bartolini, D.; Donato, F. Comparison of the inter observer reproducibility with different histologic criteria used in celiac disease. *Clin. Gastroenterol. Hepatol.* **2007**, *5*, 838–843. [CrossRef]
6. Lundin, K.E.; Scott, H.; Hansen, T.; Paulsen, G.; Halstensen, T.S.; Fausa, O.; Thorsby, E.; Sollid, L.M. Gliadin-specific, HLA-DQ(alpha 1*0501,beta 1*0201) restricted T cells isolated from the small intestinal mucosa of celiac disease patients. *J. Exp. Med.* **1993**, *178*, 187–196. [CrossRef]
7. Molberg, O.; Mcadam, S.N.; Körner, R.; Quarsten, H.; Kristiansen, C.; Madsen, L.; Fugger, L.; Scott, H.; Norén, O.; Roepstorff, P.; et al. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nat. Med.* **1998**, *4*, 713–717. [CrossRef] [PubMed]
8. Husby, S.; Koletzko, S.; Korponay-Szabo, L.; Kurppa, K.; Mearin, M.L.; Ribes-Koninckx, C.; Shamir, R.; Troncone, R.; Auricchio, R.; Castillejo, G.; et al. European society paediatric gastroenterology, hepatology and nutrition guidelines for diagnosing coeliac disease 2020. *J. Pediatr. Gastroenterol. Nutr.* **2020**, *70*, 141–156. [CrossRef]
9. Belkaid, Y.; Hand, T.W. Role of the microbiota in immunity and inflammation. *Cell* **2014**, *157*, 121–141. [CrossRef] [PubMed]
10. Nadal, I.; Donant, E.; Ribes-Koninckx, C.; Calabuig, M.; Sanz, Y. Imbalance in the composition of the duodenal microbiota of children with coeliac disease. *J. Med. Microbiol.* **2007**, *56*, 1669–1674. [CrossRef] [PubMed]
11. Di Gioia, D.; Aloisio, I.; Mazzola, G.; Biavati, B. Bifidobacteria: Their impact on gut microbiota composition and their applications as probiotics in infants. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 563–577. [CrossRef]
12. Losurdo, G.; Principi, M.; Iannone, A.; Ierardi, E.; Di Leo, A. The interaction between celiac disease and intestinal microbiota. *J. Clin. Gastroenterol.* **2016**, *50*, S145–S147. [CrossRef]
13. D'Argenio, V.; Casaburi, G.; Precone, V.; Pagliuca, C.; Colicchio, R.; Sarnataro, D.; Discepolo, V.; Kim, S.M.; Russo, I.; Sarnataro, D.; et al. Metagenomics reveals dysbiosis and a potentially pathogenic N. flavescens strain in duodenum of adult celiac patients. *Am. J. Gastroenterol.* **2016**, *111*, 879–890. [CrossRef]
14. Olivares, M.; Benítez-Páez, A.; de Palma, G.; Capilla, A.; Nova, E.; Castillejo, G.; Varea, V.; Marcos, A.; Garotte, J.A.; Polaco, I.; et al. Increased prevalence of pathogenic bacteria in the gut microbiota of infants at risk of developing celiac disease: The PROFICEL study. *Gut Microbes* **2018**, *9*, 551–558. [CrossRef]
15. Nistal, E.; Caminero, A.; Vivas, S.; Ruiz de Morales, J.M.; Sáenz de Miera, L.E.; Rodriguez-Aparicio, L.B.; Casqueiro, J. Differences in faecal bacteria populations and faecal bacteria metabolism in healthy adults and celiac disease patients. *Biochimie* **2012**, *94*, 1724–1729. [CrossRef] [PubMed]
16. Caminero, A.; Nistal, E.; Herrán, A.R.; Pérez-Andrés, J.; Ferrero, M.A.; Ayala, L.V.; Vivas, S.; Ruiz de Morales, J.M.G.; Albilos, S.M.; Casqueiro, F.J. Differences in gluten metabolism amon ghealthy volunteers, coeliac disease patients and first-degree relatives. *Br. J. Nutr.* **2015**, *114*, 1157–1167.

17. Bernardo, D.; Garrote, J.A.; Nadal, I.; León, A.J.; Calvo, C.; Fernandez-Salazar, L.; Blanco-Quiros, A.; Sanz, Y.; Arranz, E. Is it true that coeliacs do not digest gliadin? Degradation pattern of gliadin in coeliac diseasesmall intestinal mucosa. *Gut* **2009**, *58*, 886–887. [CrossRef] [PubMed]
18. De Palma, G.; Nadal, I.; Collado, M.C.; Sanz, Y. Effects of a gluten-free diet on gut microbiota and immune function in healthyadult human subjects. *Br. J. Nutr.* **2009**, *102*, 1154–1160. [CrossRef] [PubMed]
19. Bonder, M.J.; Tigchelaar, E.F.; Cai, X.; Trynka, G.; Cenit, M.C.; Hrdlickova, B.; Zhong, H.; Vatanen, T.; Gevers, D.; Wijmenga, C.; et al. The influence of a short-term gluten-free diet on the human gut microbiome. *Genome Med.* **2016**, *8*, 1–11. [CrossRef]
20. Hansen, L.B.S.; Roager, H.M.; Søndertoft, N.B.; Gøbel, R.J.; Kristensen, M.; Valles-Colomer, M.; Vieire-Silva, S.; Ibrugger, S.; Lind, M.V.; Maerkedahl, R.B.; et al. A low-gluten diet induces changes in the intestinal microbiome of healthy Danish adults. *Nat. Commun.* **2018**, *9*, 1–13.
21. Heyman, M.; Abed, J.; Lebreton, C.; Cerf-Bensussan, N. Intestinal permeability in coeliac disease: Insight into mechanisms and relevance to pathogenesis. *Gut* **2012**, *61*, 1355–1364. [CrossRef] [PubMed]
22. Raimondi, F.; Santoro, P.; Barone, M.V.; Pappacoda, S.; Barretta, M.L.; Nanayakkara, M.; Apicella, C.; Capasso, L.; Paludetto, R. Bile acids modulate tight junction structure and barrier function of CaCo-2 monolayers via EGFR activation. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2008**, *294*, G906–G913. [CrossRef]
23. Chirdo, F.G.; Auricchio, S.; Troncone, R.; Barone, M.V. The gliadin p31-43 peptide: Inducer of multiple proinflammatory effects. *Int. Rev. Cell Mol. Biol.* **2021**, *358*, 165–205.
24. Nilsen, E.M.; Jahnsen, F.L.; Lundin, K.E.; Johansen, F.E.; Fausa, O.; Sollid, L.M.; Jahnsen, J.; Scott, H.; Brandtzaeg, P. Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. *Gastroenterology* **1998**, *115*, 551–563. [CrossRef]
25. Beckett, C.G.; Dell'Olio, D.; Shidrawi, R.G.; Rosen-Bronson, S.; Ciclitira, P.J. Gluten-induced nitric oxide and pro-inflammatory cytokine release by cultured coeliac small intestinal biopsies. *Eur. J. Gastroenterol. Hepatol.* **1999**, *11*, 529–535. [CrossRef]
26. Ventura, M.; Canchaya, C.; Fitzgerald, G.F.; Gupta, R.S.; van Sinderen, D. Genomics as a means to understand bacterial phylogeny and ecological adaptation: The case of bifidobacteria. *Antonie Leeuwenhoek* **2007**, *91*, 351–372. [CrossRef]
27. Ventura, M.; Turroni, F.; Canchaya, C.; Vaughan, E.E.; O'Toole, P.W.; van Sinderen, D. Microbial diversity in the human intestine and novel insights from metagenomics. *Front. Biosci.* **2009**, *14*, 3214–3221. [CrossRef]
28. Ventura, M.; Turroni, F.; Lugli, G.A.; van Sinderen, D. Bifidobacteria and humans: Our special friends, from ecological to genomics perspectives. *J. Sci. Food Agric.* **2014**, *94*, 163–168. [CrossRef]
29. Tannock, G.W. Identification of lactobacilli and bifidobacteria. *Curr. Issues Mol. Biol.* **1999**, *1*, 53–64.
30. Laparra, J.M.; Sanz, Y. Bifidobacteria inhibit the inflammatory response induced by gliadins in intestinal epithelial cells via modifications of toxic peptide generation during digestion. *J. Cell Biochem.* **2010**, *109*, 801–807. [CrossRef]
31. Olivares, M.; Laparra, M.; Sanz, Y. Oral administration of Bifidobacterium longum CECT 7347 modulates jejunal proteome in an in vivo gliadin-induced enteropathy animal model. *J. Proteom.* **2012**, *77*, 310–320. [CrossRef]
32. De Almeida, N.E.C.; Esteves, F.G.; Dos Santos-Pinto, J.R.A.; Peres de Paula, C.; da Cunha, A.F.; Malavazi, I.; Palma, M.S.; Rodrigues-Filho, E. Digestion of intact gluten proteins by bifidobacterium species: Reduction of cytotoxicity and proinflammatory responses. *J. Agric. Food Chem.* **2020**, *68*, 4485–4492. [CrossRef] [PubMed]
33. Giorgi, A.; Cerrone, R.; Capobianco, D.; Filardo, S.; Mancini, P.; Zanni, F.; Fanelli, S.; Mastromarino, P.; Mosca, L. A probiotic preparation hydrolyzes gliadin and protects intestinal cellsfrom the toxicity of pro-inflammatory peptides. *Nutrients* **2020**, *12*, 495. [CrossRef] [PubMed]
34. Heeney, D.D.; Gareau, M.G.; Marco, M.L. Intestinal Lactobacillus in health and disease, a driver or just along for the ride? *Curr. Opin. Biotechnol.* **2018**, *49*, 140–147. [CrossRef]
35. Orlando, A.; Linsalata, M.; Notarnicola, M.; Tutino, V.; Russo, F. Lactobacillus GG restoration of the gliadin induced epithelial barrier disruption: The role of cellular polyamines. *BMC Microbiol.* **2014**, *14*, 1–12. [CrossRef] [PubMed]
36. Orlando, A.; Linsalata, M.; Bianco, G.; Notarnicola, M.; D'Attoma, B.; Scavo, M.P.; Tafaro, A.; Russo, F. Lactobacillus rhamnosus GG protects the epithelial barrier of wistar rats from the pepsin-trypsin-digested gliadin (PTG)-induced enteropathy. *Nutrients* **2018**, *10*, 1698. [CrossRef]
37. Barone, M.V.; Nanayakkara, M.; Paolella, G.; Maglio, M.; Vitale, V.; Troiano, R.; Ribecco, M.T.S.; Lania, G.; Zanzi, D.; Santagata, S.; et al. Gliadin peptide P31-43 localises to endocytic vesicles and interferes with their maturation. *PLoS ONE* **2010**, *5*, e12246. [CrossRef]
38. Barone, M.V.; Zanzi, D.; Maglio, M.; Nanayakkara, M.; Santagata, S.; Lania, G.; Miele, E.; Ribecco, M.T.S.; Maurano, F.; Auricchio, R.; et al. Gliadin-mediated proliferation and innate immune activation in celiac disease are due to alterations in vesicular trafficking. *PLoS ONE* **2011**, *6*, e17039.
39. Zimmer, K.-P.; Fischer, I.; Mothes, T.; Weissen-Plenz, G.; Schmitz, M.; Wieser, H.; Büning, J.; Lerch, M.M.; Ciclitira, P.C.; Weber, P.; et al. Endocytotic segregation of gliadin peptide 31–49 in enterocytes. *Gut* **2009**, *59*, 300–310. [CrossRef]
40. Sarno, M.; Lania, G.; Cuomo, M.; Nigro, F.; Passannanti, F.; Budelli, A.; Fasano, F.; Troncone, R.; Auricchio, S.; Barone, M.V.; et al. Lactobacillus paracaseiCBA L74 interferes with gliadin peptides entrance in Caco-2 cells. *Int. J. Food Sci. Nutr.* **2014**, *65*, 953–959. [CrossRef]
41. Gibson, G.R.; Roberfroid, M.B. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. *J. Nutr.* **1995**, *125*, 1401–1412. [CrossRef]

42. Roberfroid, M. Functional food concept and its application to prebiotics. *Dig. Liver Dis.* **2002**, *34*, S105–S110. [CrossRef]
43. Chen, Q.; Ren, Y.; Lu, J.; Bartlett, M.; Chen, L.; Zhang, Y.; Guo, X.; Liu, C. A Novel prebiotic blend product prevents irritable bowel syndrome in mice by improving gut microbiota and modulating immune response. *Nutrients* **2017**, *9*, 1341. [CrossRef] [PubMed]
44. Iaffaldano, L.; Granata, I.; Pagliuca, C.; Esposito, M.V.; Casaburi, G.; Salerno, G.; Colicchio, R.; Piccirillo, M.; Ciacci, C.; Blanco, G.D.V.; et al. Oropharyngeal microbiome evaluation highlights Neisseria abundance in active celiac patients. *Sci. Rep.* **2018**, *8*, 1–10. [CrossRef]
45. Labruna, G.; Nanayakkara, M.; Pagliuca, C.; Nunziato, M.; Iaffaldano, L.; D'Argenio, V.; Colocchio, R.; Budelli, A.L.; Nigro, R.; Salvatore, P.; et al. Celiac disease-associated Neisseria flavescens decreases mitochondrial respiration in CaCo-2 epithelial cells: Impact of Lactobacillus paracasei CBA L74 on bacterial-induced cellular imbalance. *Cell Microbiol.* **2019**, *21*, e13035. [CrossRef] [PubMed]
46. Gallo, M.; Nigro, F.; Passannanti, F.; Nanayakkara, M.; Lania, G.; Parisi, F.; Salameh, D.; Budelli, A.; Barone, M.V.; Nigro, R. Effect of pH control during rice fermentation in preventing a gliadin P31-43 entrance in epithelial cells. *Int. J. Food Sci. Nutr.* **2019**, *70*, 950–958. [CrossRef] [PubMed]
47. Freire, R.; Ingano, L.; Serena, G.; Cetinbas, M.; Anselmo, A.; Sapone, A.; Sadreyev, R.I.; Fasano, A.; Senger, S. Human gut derived-organoids provide model to study gluten response and effects of microbiota-derived molecules in celiac disease. *Sci. Rep.* **2019**, *9*, 1–15. [CrossRef] [PubMed]
48. Verdu, E.F.; Galipeau, H.; Jabri, B. Novel players in coeliac disease pathogenesis: Role of the gut microbiota. *Nat. Rev. Gastroenterol. Hepatol.* **2015**, *12*, 497–506. [CrossRef]
49. Pagliari, D.; Urgesi, R.; Frosali, S.; Riccioni, M.E.; Newton, E.E.; Landolfi, R.; Pandolfi, F.; Cianci, R. The interaction among microbiota, immunity, and genetic and dietary factors is the condicio sine qua non celiac disease can develop. *J. Immunol. Res.* **2015**, *48*, 123653.
50. de Graaf, A.A.; Venema, K. Gaining insight into microbial physiology in the large intestine: A special role for stable isotopes. *Adv. Microb. Physiol.* **2008**, *53*, 73–314.
51. Olivares, M.; Castillejo, G.; Varea, V.; Sanz, Y. Double-blind, randomised, placebo-controlled intervention trial to evaluate the effects of Bifidobacterium longum CECT 7347 in children with newly diagnosed coeliac disease. *Br. J. Nutr.* **2014**, *112*, 30–40. [CrossRef]
52. Klemenak, M.; Dolinšek, J.; Langerholc, T.; Di Gioia, D.; Mičetić-Turk, D. Administration of bifidobacterium breve decreases the production of TNF-α in children with celiac disease. *Dig. Dis. Sci.* **2015**, *60*, 3386–3392. [CrossRef] [PubMed]
53. Quagliariello, A.; Aloisio, I.; Cionci, N.B.; Luiselli, D.; D'Auria, G.; Martinez-Priego, L.; Pérez-Villarroya, D.; Langerholc, T.; Primec, M.; Mičetić-Turk, D.; et al. Effect of bifidobacterium breve on the intestinal microbiota of coeliac children on a gluten free diet: A pilot study. *Nutrients* **2016**, *8*, 660. [CrossRef]
54. Harnett, J.; Myers, S.P.; Rolfe, M. Probiotics and the microbiome in celiac disease: A randomised controlled trial. *Evid.-Based Complement. Altern. Med.* **2016**, *2016*, 1–16. [CrossRef] [PubMed]
55. Francavilla, R.; Piccolo, M.; Francavilla, A.; Polimeno, L.; Semeraro, F.; Cristofori, F.; Castellaneta, S.; Barone, M.; Indrio, F.; Gobbetti, M.; et al. Clinical and microbiological effect of a multispecies probiotic supplementation in celiac patients with persistent IBS-type Symptoms: A randomized, double-blind, placebo-controlled, multicenter trial. *J. Clin. Gastroenterol.* **2019**, *53*, e117–e125. [CrossRef] [PubMed]
56. Smecuol, E.; Hwang, H.J.; Sugai, E.; Corso, L.; Cherñavsky, A.C.; Bellavite, F.P.; González, A.; Vodánovich, F.; Moreno, M.L.; Vázquez, H.; et al. Exploratory, randomized, double-blind, placebo-controlled study on the effects of bifidobacterium infantis natren life start strain super strain in active celiac disease. *J. Clin. Gastroenterol.* **2013**, *47*, 139–147. [CrossRef]
57. Pinto-Sánchez, M.I.; Smecuol, E.C.; Temprano, M.P.; Sugai, E.; González, A.; Moreno, M.L.; Huang, X.; Bercik, P.; Cabanne, A.; Vaszquez, H.; et al. Bifidobacterium infantis NLS super strain reduces the expression of α-defensin-5, a marker of innate immunity, in the mucosa of active celiac disease patients. *J. Clin. Gastroenterol.* **2017**, *51*, 814–817. [CrossRef] [PubMed]
58. Hogen Esch, C.E.; Rosén, A.; Auricchio, R.; Romanos, J.; Chmielewska, A.; Putter, H.; Ivarsson, A.; Szajewska, H.; Koning, F.; Wijmenga, C.; et al. Prevent CD Study Group. The PreventCD study design: Towards new strategies for the prevention of coeliac disease. *Eur. J. Gastroenterol. Hepatol.* **2010**, *22*, 1424–1430. [PubMed]
59. Lionetti, E.; Castellaneta, S.; Francavilla, R.; Pulvirenti, A.; Tonutti, E.; Amarri, S.; Barbato, M.; Barbera, C.; Barera, G.; Bellantoni, A.; et al. Introduction of gluten, hla status, and the risk of celiac disease in children. *N. Engl. J. Med.* **2014**, *371*, 1295–1303. [CrossRef]
60. Uusitalo, U.; Aronsson, C.A.; Liu, X.; Kurppa, K.; Yang, J.; Liu, E.; Skidmore, J.; Winkler, C.; Rewers, M.J.; Hagopian, W.A.; et al. Early probiotic supplementation and the risk of celiac disease in children at genetic risk. *Nutrients* **2019**, *11*, 1790. [CrossRef]
61. Håkansson, A.; Aronsson, C.A.; Brundin, C.; Oscarsson, E.; Molin, G.; Agardh, D. Effects of lactobacillus plantarum and lactobacillus paracasei on the peripheral immune response in children with celiac disease autoimmunity: A randomized, double-blind, placebo-controlled clinical trial. *Nutrients* **2019**, *11*, 1925. [CrossRef]
62. Gibson, G.R.; Hutkins, R.; Sanders, M.E.; Prescott, S.L.; Reimer, R.A.; Salminen, S.J.; Scott, K.; Stanton, C.; Swanson, K.S.; Cani, P.D.; et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat. Rev. Gastroenterol. Hepatol.* **2017**, *14*, 491–502. [CrossRef] [PubMed]

63. Feruś, K.; Drabińska, N.; Krupa-Kozak, U.; Jarocka-Cyrta, E. A randomized, placebo-controlled, pilot clinical trial to evaluate the effect of supplementation with prebiotic synergy 1 on iron homeostasis in children and adolescents with celiac disease treated with a gluten-free diet. *Nutrients* **2018**, *10*, 1818. [CrossRef] [PubMed]
64. Husby, S.; Koletzko, S.; Korponay-Szabó, I.R.; Mearin, M.L. European Society for Pediatric Gastroenterology, Hepatology, and Nutrition guidelines for the diagnosis of coeliac disease. *J. Pediatr. Gastroenterol. Nutr.* **2012**, *54*, 136–160. [CrossRef]
65. Drabińska, N.; Jarocka-Cyrta, E.; Markiewicz, L.H.; Krupa-Kozak, U. The effect of oligofructose-enriched inulin on faecal bacterial counts and microbiota-associated characteristics in celiac disease children following a gluten-free diet: Results of a randomized, placebo-controlled trial. *Nutrients* **2018**, *10*, 201. [CrossRef]
66. Drabińska, N.; Krupa-Kozak, U.; Abramowicz, P.; Jarocka-Cyrta, E. Beneficial effect of oligofructose-enriched inulin on vitamin d and e status in children with celiac disease on a long-term gluten-free diet: A preliminary randomized, placebo-controlled nutritional intervention study. *Nutrients* **2018**, *10*, 1768. [CrossRef] [PubMed]
67. Drabińska, N.; Krupa-Kozak, U.; Coska, E.; Jarocka-Cyrta, E. Plasma profile and urine excretion of amino acids in children with celiac disease on gluten-free diet after oligofructose-enriched inulin intervention: Results of a randomised placebo-controlled pilot study. *Amino Acids* **2018**, *50*, 1451–1460. [CrossRef]
68. Drabińska, N.; Jarocka-Cyrta, E.; Złotkowska, D.; Abramowicz, P.; Krupa-Kozak, U. Daily oligofructose-enriched inulin intake impacts bone turnover markers but not the cytokine profile in pediatric patients with celiac disease on a gluten-free diet: Results of a randomised, placebo-controlled pilot study. *Bone* **2019**, *122*, 184–192. [CrossRef]

International Journal of
Molecular Sciences

MDPI

.Type of the Paper

Inflammation is present, persistent and more sensitive to pro-inflammatory triggers in celiac disease enterocytes

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Abstract: Celiac disease (CD) is a chronic inflammatory disease caused by a genetic predisposition to an abnormal T cell mediated immune response to the gluten in the diet. Different environmental proinflammatory factors can influence and amplify the T cell mediated response to gluten. The aim of this manuscript was to study the role of enterocytes in CD intestinal inflammation and the response to different proinflammatory factors, such as gliadin and viruses.Intestinal biopsies from CD patients on gluten-containing (GCD-CD) or gluten-free diet (GFD-CD) as well as biopsies from potential CD patients (Pot-CD) before the onset of intestinal lesions and controls (CTR) were used to investigate IL-1β and IL-6 mRNA levels in situ. Organoids from CD patients were used to test the levels of NF-κB, ERK, IL-6, and IL-1β by Western blot (WB), ELISA and quantitative PCR. The Toll-like receptor ligand loxoribine (Lox) and gliadin peptide P31-43 were used as proinflammatory stimuli.In CD biopsies inflammation markers IL-1β and IL-6 were increased in the enterocytes, also in Pot-CD before the onset of the intestinal lesion and in GFD-CD. The inflammatory markers pNF-κB, pERK, IL-1β and IL-6 were increased and persistent in CD organoids; these organoids were more sensitive to P31-43 and Lox stimuli compared with CTR organoids.Taken together, these observations point to constitutive inflammation in CD enterocytes, which are more sensitive to inflammatory stimuli such as food components and viruses.

Synopsis

Inflammation is present in CD enterocytes even before the onset of intestinal lesions, even with a gluten-free diet.

Organoids derived from CD intestinal stem cells reproduced the intestinal inflammation.

CD enterocytes were more sensitive to proinflammatory stimuli.

Keywords: Small intestine; Potential Celiac Disease; NF-κB; ERK

Citation: Porpora M1; Conte M1; Lania G1; Bellomo C1; Rapacciulo L1; Chirdo F2; Auricchio R1; Troncone R1; M V, B.; Nanayakkara M1 Inflammation is present, persistent and more sensitive to proinflammatory triggers in celiac disease enterocytes. *Int. J. Mol. Sci.* **2021**, *22*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s):

Received: date
Accepted: date
Published: date

1. Introduction

Celiac disease (CD) is an immune-mediated enteropathy triggered in genetically susceptible individuals by a group of wheat proteins and related prolamins from cereals [1]. The HLA-restricted gliadin-specific intestinal T cell response plays a central role in the pathogenesis of CD [1]. CD is known to be characterized by a combination of gluten-induced symptoms, the generation of CD-associated autoantibodies, and enteropathy [2], but it remains unclear why T cells are activated by gliadin. Studies conducted in mice have demonstrated that mucosal inflammation due to reovirus infection may disrupt oral toleranc to gliadin by suppressing regulatory T cell conversion and promoting Th1 immunity [3]. These results indicate that in an inflamed environment enriched in cytokines, T cells tend to acquire a proinflammatory phenotype. The factors that create a proinflammatory environment in the CD intestine could have multiple origins. Recent studies have de-

scribed the influence of several different factors in CD, such as cellular vulnerability, the proinflammatory effects of gluten [4,5] and other wheat proteins [6], Western diet [7] and other environmental triggers, such as viruses [3,8] that may prepare and/or amplify, the TC -mediated response to gluten [9].

Cellular vulnerability in CD has been suggested by recent studies that have shown that despite clinical and histological remission, celiac disease patients fed a gluten-free diet (GFD) have altered protein composition of gut tissue with signs of ongoing inflammation. In fact, changes indicative of epithelial inflammation, minor crypt hyperplasia and in the serum have low-grade inflammation been demonstrated in GFD-CD intestinal biopsies before gluten challenge in vivo [10]. Moreover, alterations in differentiation/proliferation pathways have been found in GFD-CDpatients before in vivo challenge with gluten [11]. Interestingly, after in vivo challenge with gluten, an increase in the same proteins that were altered at baseline in the GFD-CD, was found [10,11]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a major mediator of the inflammatory response [12], has been found to be increased in GCD-CD and GFD-CD biopsies from several different genetic [13] and expression studies[14-16]. Fernandez-Jimenez et al. [14] showed that the expression of93 NF-κB genes measured by RT–PCR (real-time polymerase chain reaction) was altered in GFD-CD and GCD-CD compared to control biopsies and in cultured biopsies from CD (GCD and GFD) patients challenged with gliadin. These results showed that genes that were constitutively upregulated in GFD patients belonged to the NF-κB signalling system. Interestingly, according to GFD biopsies, gluten challenge also increased NF-κB pathway activation in vitro. Similar increases in the activation of the NF-κB pathway were observed in GFD-CD biopsies and fibroblasts, both derived from the intestines of CD patients and from skin explants (distant from the intestine). Only biopsies and fibroblasts from CD patients were sensitive to low doses of gliadin peptide P31-43, which did not affect controls [17]. Taken together, these data indicate that in CD, a vulnerability (probably constitutive) is present that renders cells more sensitive to proinflammatory stimuli.

The intestinal epithelium has assumed an emerging role in CD pathogenesis; in fact, morphological [4,17] and functional alterations [17,18] have been described in epithelial cells in CD patients. Moreover, the inflammasome pathway was increased in intestinal epithelial cells isolated from GFD-CD biopsies after gluten challenge [19].

Gluten and, in particular, one of its undigested peptides (P31-43) can induce inflammation in cultured cells, in CD biopsies (GCD and GFD) and in mice [5]. P31-43 is not presented to T cells and can induce several different effects on cells, in CD biopsies and mice, including proliferation, activation of innate immune markers and inflammation [5,20]. Moreover, it can act synergistically with viral ligands (Poli I:C and loxoribine) to induce innate immunity activation both in vitro and in vivo [21]

Therefore, the emerging role of epithelial inflammation and the proinflammatory effects of viruses and gliadin in CD prompted us to study inflammatory markers in situ in the enterocytes of biopsies derived from CD patients at different stages of the disease (GCD-, GFD- and Pot-CD). Moreover, we examined whether intestinal organoids derived from CD biopsies are a good model for studying inflammation and sensitivity to proinflammatory agents, such as P31-43 and the viral ligand loxoribine, in CD epithelium.

2. Results

2.1. In CD biopsies, the inflammatory markers IL-1β and IL-6 are increased in enterocytes.

Expression of IL-1β and IL-6 mRNA in situ was analysed in intestinal biopsies from CD patients at different stages of the disease as well as those from controls. IL-1β mRNA was absent in control biopsies at both the level of the intestinal epithelial cells of the crypts (Figure 1 A and E) and of the villi (Supplemental Figure 1A). In contrast, IL-1β mRNA was increased in all CD patients independent of the stage of the disease and almost exclusively at the level of the crypts (Figure 1 B-D; Supplemental Figure 1 B-E). In particular, IL-1β mRNA was highly expressed (59±36 red dots/crypt, p value <0.05) in CD patients fed a gluten-containing diet (GCD-CD) (Figure 1 B, E) in the acute phase of the disease and in potential patients (Pot-CD) (43.6 ± 11 red dots/crypt, p value <0,01) (Figure 1 C, E) before the onset of lesions of the small intestine, who still consumed gluten. Patients in the remission phase of the disease (GFD-CD) on a gluten-free diet also presented an increased expression of IL-1β in the intestinal epithelium, although at lower levels, than patients in the GCD-CD and Pot-CD groups (17.6 ± 2.5 red dots/crypt, p value <0,001) (Figure 1 D, E).

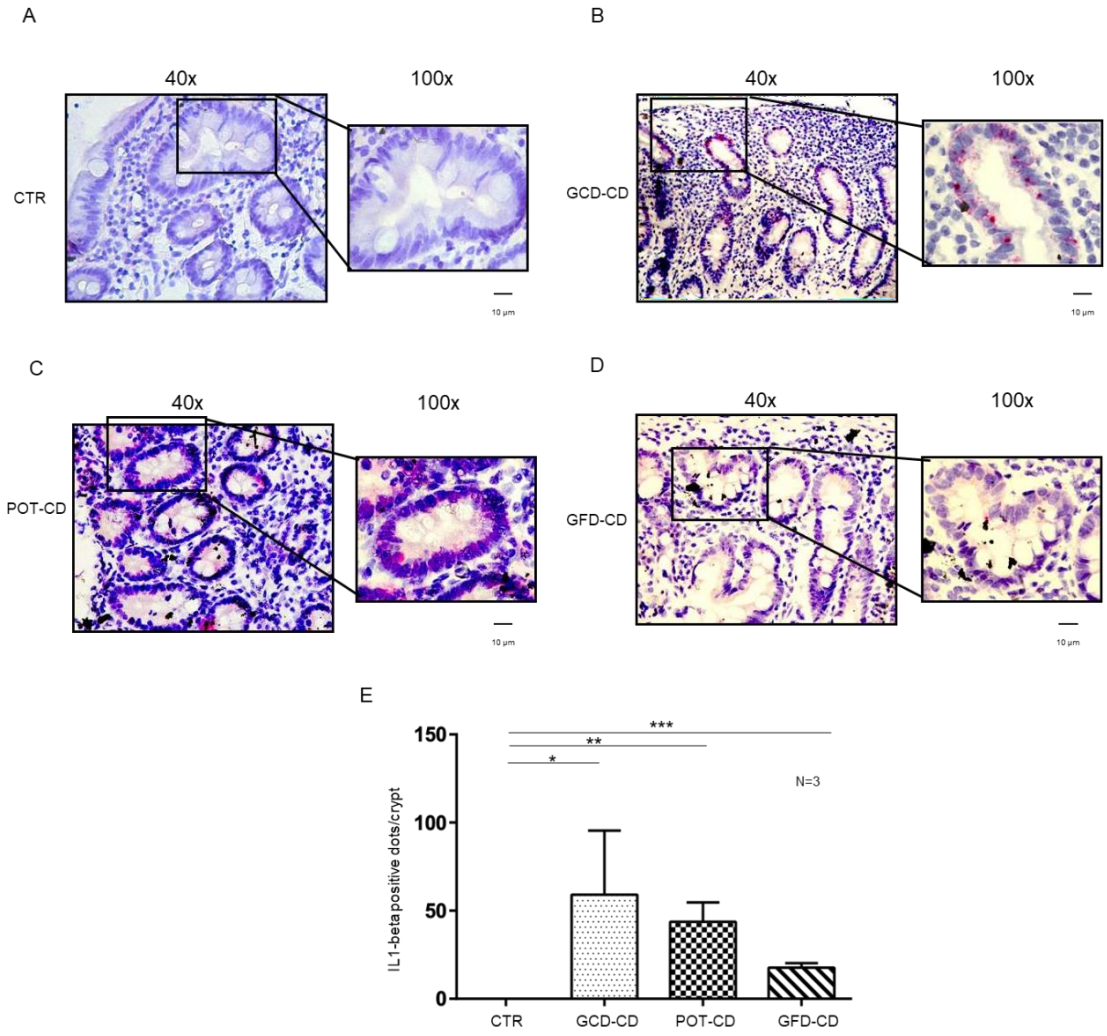
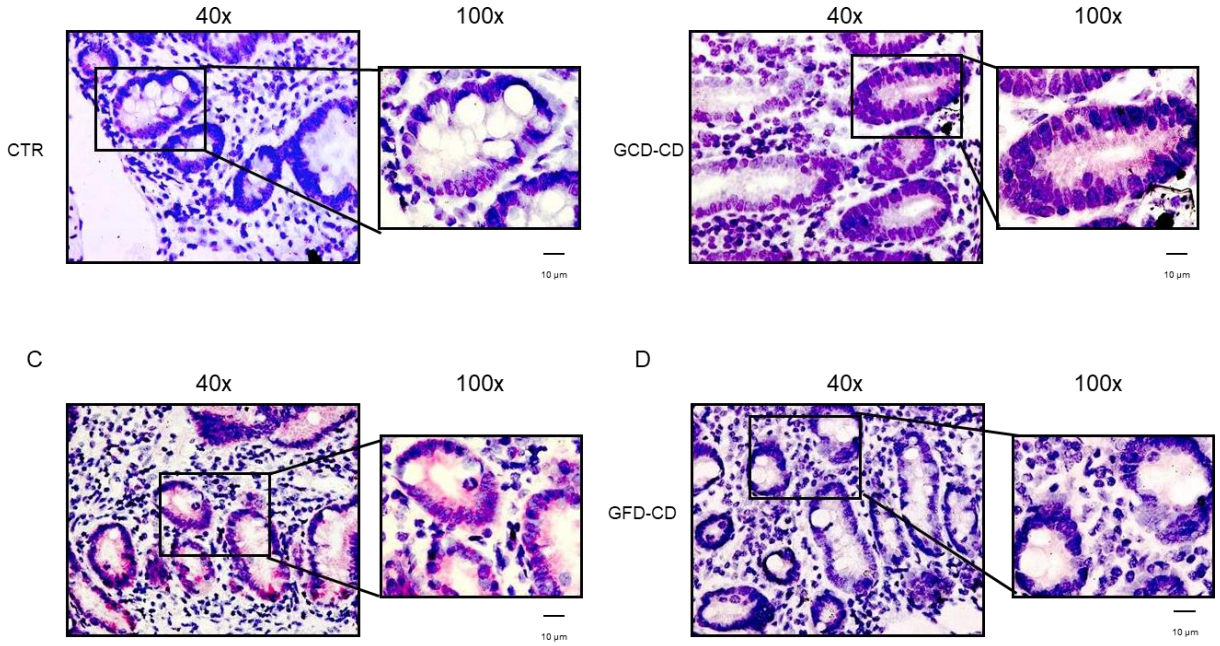


Figure 1. IL-1β is increased in the epithelium of the crypts in CD biopsies. In situ mRNA analysis of IL-1βeta in biopsies from controls (CTR) (A), gluten-containing diet celiac disease patients (GCD-CD) (B), Potential CD patients (Pot-CD) (C), and gluten-free diet celiac patients (GFD-CD) (D).Blue indicates haematoxylin-eosin staining of the nuclei, and red indicates IL-1β mRNA. Black squares show different enlargements of crypts. Lines indicate 10 micrometers at 100X objective.

E. IL-1β-positive red dot counts in the crypts of intestinal biopsies from CD patients and controls. At least 10 crypts/subject were counted on different slides. The numbers of subjects analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

IL-6 mRNA was present in control biopsies both at the level of the crypts (Figure 2 A and E) and of the villi(Supplemental Figure 2 A), as expected[22]. On the other hand, IL-6, similar to IL-1β (Figure 2), was overexpressed with respect to the control in the GCD-CD, GFD-CD and Pot-CD groups (Figure 2 B-D).

In particular, IL-6 mRNA was highly expressed (42.5±8.6 red dots/crypt) in CD patients fed a gluten-containing diet (GCD-CD) (Figure 2 B, E; *p* value <0,01) in the acute phase of the disease and in potential patients (Pot-CD) (53.67± 26.7 red dots/crypt; *p* value <0,05) (Figure 2 C, E) before the onset of lesions of the small intestine, who still consumed gluten. Patients in the remission phase of the disease and fed a gluten-free diet (GFD-CD) (Figure 2D) also presented altered expression of IL-6 in the intestinal epithelium, although the difference did not reach statistical significance (30.67± 29.01red dots/crypt) (Figure 2 D, E).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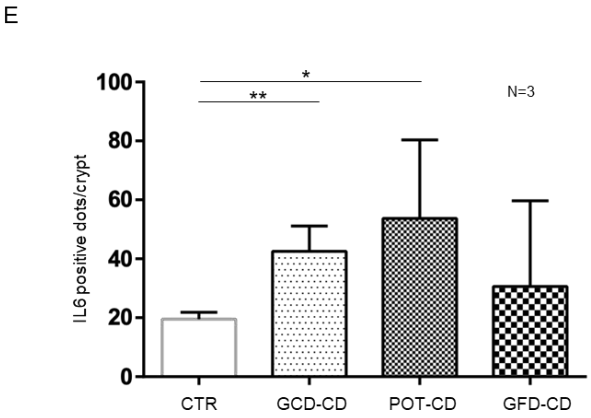
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Figure 2. IL-6 levels were increased in the epithelium of the crypts in CD biopsies. In situ mRNA analysis of IL-6 in biopsies from controls (CTR) (A), gluten-containing diet celiac disease patients (GCD-CD) (B), Potential CD patients (Pot-CD) (C), and gluten-free diet celiac patients (GFD-CD) (D). Blue indicates haematoxylin-eosin staining of the nuclei, and red indicates IL-6 mRNA. Black squares show different enlargements of crypts. Lines indicate 10 micrometers at 100X objective. E. IL-6-positive red dot counts in the crypts of intestinal biopsies from CD patients and controls. At least 10 crypts/subject were counted on different slides. The numbers of subjects analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: * = $p<0.05$; ** = $p<0.01$.

2.2 The inflammatory markers pNF-κB, pERK, IL-1β, and IL-6 were increased and persistent in CD organoids.

Intestinal organoids were derived from GCD-CD and CTR intestinal biopsiesand tested after 1 week of culture for the inflammatory markers pNF-κB, pERK, IL-1β, and IL-6. In particular, pNF-κB (*p* value <0.05) and pERK (*p* value <0.05) were increased in CD organoids compared to CTR organoids, as evaluated by Western blot (WB) analysis (Figure 3 A-D). Quantitative mRNA (Figure 3 E and F) showed a marked increase in IL-1β (*p* value <0.05) and IL-6 (*p* value <0.01) expression in CD organoids compared with that of CTR organoids. An ELISA of the supernatant of the organoid cultures (Figure 3 G and H) showed a marked increase in IL-1β (*p* value <0.05) and IL-6 (*p* value <0.01) levels in the CD supernatant compared with those of CTR supernatant.

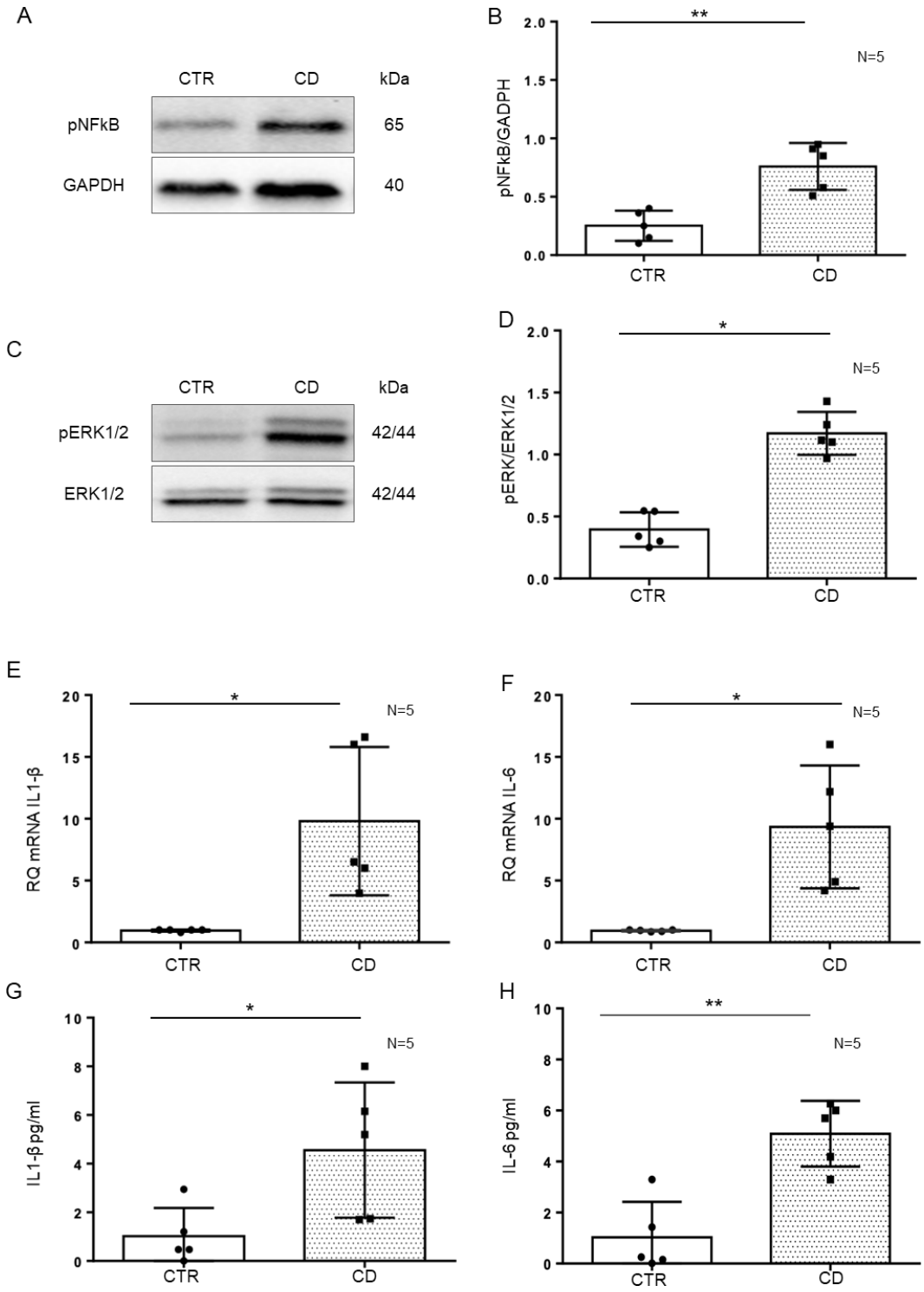


Figure 3 Inflammatory markers were increased in CD organoids. A. Western blot analysis of total protein lysates of organoids from controls (CTR) and CD patients fed a gluten-containing diet (CD).

Upper lines were blotted with an antibody against the phosphorylated form of NF-κB (pNF-κB). Bottom lines were blotted with anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody as a loading control. Representative images were selected. B. Densitometric analysis of the pNF-κB/GAPDH bands from CTR and CD. The numbers of organoids analysed are indicated. Col- umns represent the mean, and bars represent the standard deviation. Student's *t test*: ** = p<0.01. C. Western blot analysis of total protein lysates of organoids from CTR and CD. Upper lines were blottedwith an antibody against the phosphorylated form of ERK (pERK). Bottom lines were blotted with an-ti-ERK antibody as a loading control. Representative images were selected. D. Densitometric analysis of the pERK/ERK bands from CTR and CD. The numbers of organoids analysed are indicated. Col- umns represent the mean, and bars represent the standard deviation. Student's *t test*: * = p<0.05. E and F. Quantitative PCR analysis of IL-1β and IL-6 mRNA levels in CD patients compared to CTR patients. The number of organoids analysed is indicated. The columns represent the mean, and bars represent the standard deviation. Student's *t test*: * = p<0.05. G and H. ELISA showing IL-1β and IL-6 protein levels in the culture media of CD organoids compared to CTR organoids. The numbers of organoids are indicated. The columns represent the mean, and bars represent the standard deviation. Student's *t test*: * =p<0.05; ** =p<0.01.

To understand whether the inflammation in intestinal organoids was a residual effect of the inflamed intestinal environment or inherent to the intestinal CD epithelium, we cultivated organoids from CD

and CTR patients for several weeks (from 4 to 12 weeks) and then tested them again for the inflammatory markers pNF-κB and pERK by WB analysis (Figure 4 A-C). Interestingly, the expression of the inflammatory markers pNF-κB (p value <0.01) and pERK (p value <0.01) in CD organoids remained more elevated than in CTR organoids after several weeks in culture .

In intestinal organoids from inflammatory bowel disease (IBD) patients, markers of inflammation are elevated only for a few weeks (1-2 w) after culture and then decrease to the level of CTR organoids.[23] In Figure 4 D-F, we compared the inflammatory markers pNF-κB and pERK in intestinal organoids from CD and IBD patients after 4 weeks in culture. We confirmed that in IBD, both pNF-κB and pERK were at the level of the controls. Only CD organoids showed higher levels of pNF-κB (p value <0.01) and pERK (p value <0.05), than the CTR organoids after 4 weeks in culture. Taken together, this indicates that in CD, contrary to IBD, inflammation is persistent.

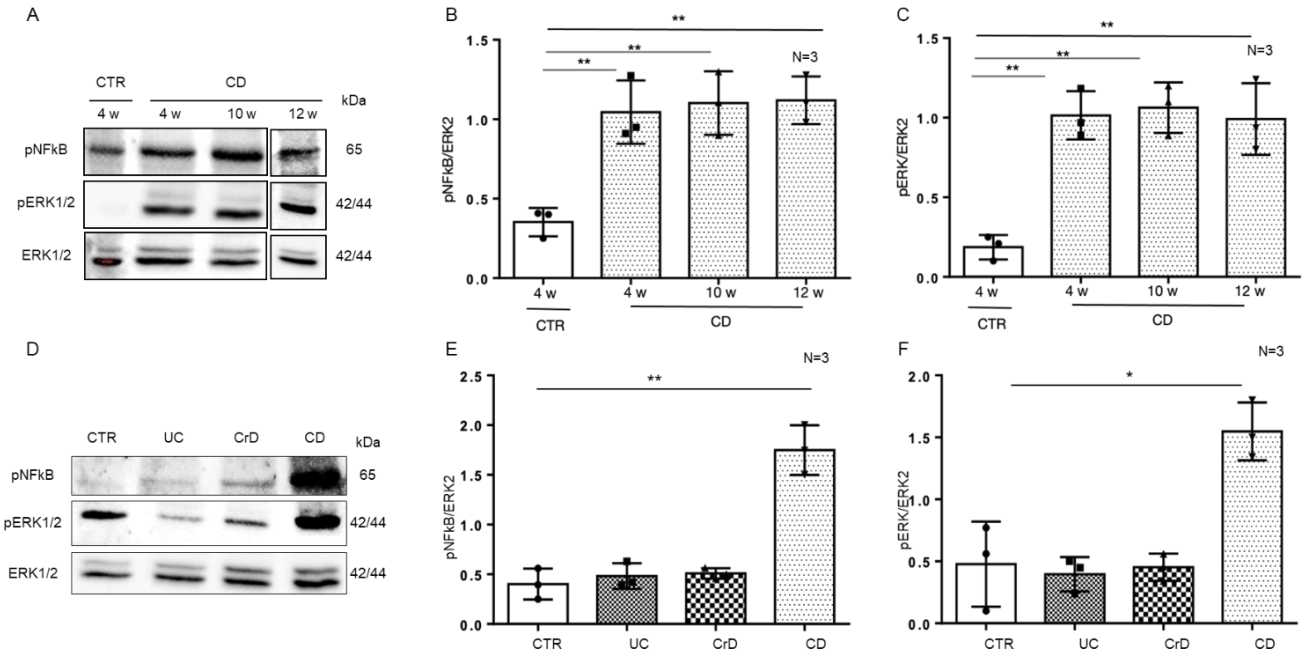


Figure 4. Inflammation was persistent in CD organoids. A. Western blot analysis of the total protein lysates of organoids from CD patients cultivated for several weeks as indicated. Upper lines were

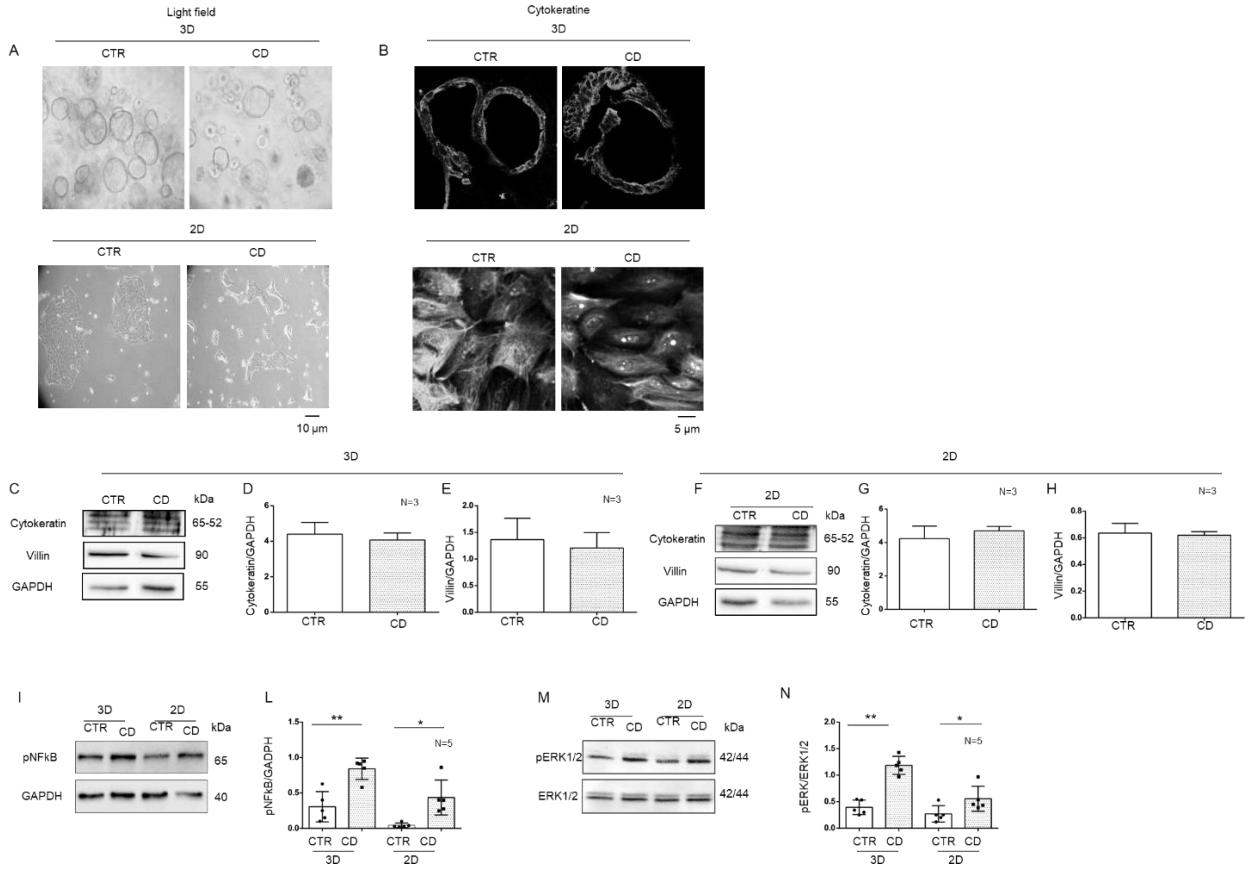
blotted with an antibody against the phosphorylated form of NF-κB (pNF-κB). Middle lines were blotted with an antibody against the phosphorylated form of ERK (pERK). Bottom lines were blotted with anti-ERK antibody as a loading control. Representative images were selected. B. Densitometric analysis of the pNF-κB/ERK bands from CD cultivated for different times as indicated. The numbers of subjects analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: * = $p<0.01$. C. Densitometric analysis of the pERK/ERK bands from CD cultivated for different times as indicated. The numbers of subjects analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: ** = $p<0.01$.

D. Western blot analysis of total protein lysates of organoids from CTR, ulcerative colitis (UC), Crohn's disease (CrD) and CD cultivated for 4 weeks. Upper lines were blotted with an antibody against the phosphorylated form of NF-κB (pNF-κB). Middle lines were blotted with an antibody against the phosphorylated form of ERK (pERK). Bottom lines were blotted with anti-ERK antibody as a loading control. Representative images were selected. E. Densitometric analysis of the pNF-κB/ERK bands from CTR, UC, CrD and CD cultivated for 4 weeks. The numbers of subjects analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: ** = $p<0.01$. F. Densitometric analysis of the pERK/ERK bands from CTR, UC, CrD and CD cultivated for 4 weeks. The numbers of subjects analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: * = $p<0.05$.

2.3 3D and 2D organoids from CD patients had increased inflammatory markers compared to those from CTR patients

Organoids are considered good models for studying inflammation and infection of the intestine [24]. To examine the role of inflammation in CD, we treated CD organoids with proinflammatory agents such as gliadin peptide P43-43 and the viral ligand loxoribine (Lox), which is a specific ligand for TLR7 (Toll-like receptor 7) [21]. Previous data indicated that both P31-43 and Lox increased inflammatory markers in CD cells (8).

To provide correct treatment for the apical side of the intestinal cells of organoids, it is necessary to open them up, shifting them to 2D because in 3D, the apical side of the cells is enclosed in the spherical organoids. Light microscopy analysis of organoids in 3D and 2D did not show any difference in their dimensions. Three-dimensional CD organoids were denser than CTR organoids, as already described [25](Figure 5 A). The expression of villin and cytokeratin, differentiation markers of epithelial cells, was similar in 3D and 2D organoids, as assessed by immunofluorescence and WB (Figure 5 B-H). Phosphorylation levels of NF-κB and ERK in 3D organoids were compared to those in 2D organoids, for both CD and CTR patients (Figure 5 I-N). pNF-κB was increased in both 2D (p value <0.05) and 3D (p value <0.01) CD organoids compared to CTR organoids. Additionally, pERK was increased in both 2D (p value <0.05) and 3D (p value <0.01) CD organoids compared to CTR organoids. We confirmed that opening the organoids did not affect inflammatory marker expression (Figure 5 I-N).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3D
CTR
CD
2D
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CD
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Cytokeratine
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3D
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65-52
Villin
90
GAPDH
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Figure 5.Markers of inflammation were increased in both 3D and 2D organoids from CD patients compared to CTR patients. A. Light field microscopy of 3D and 2D organoids from CD and CTR. 20X objective. The black line indicates 10 microns. B Immunofluorescence images of 3D and 2D organoids stained with anti-cytokeratin antibodies. 60x objective.The black line indicates 5 microns. C. Western blot analysis of total protein lysates from CTR and CD 3D organoids. Upper lines were blotted with an antibody against cytokeratin. Middle lines were blotted with an antibody against villin. Bottom lines were blotted with GAPDH antibody as a loading control. Representative images were selected. Densitometric analysis of the cytokeratine/GAPDH (D) and villin/GAPDH (E) bands from 3D organoids from CTR and CD. Columns represent the mean, and bars represent the standard deviations. The numbers of subjects analysed are indicated.

F. Western blot analysis of total protein lysates from CTR and CD 2D organoids. Upper lines were blotted with an antibody against cytokeratin. Middle lines were blotted with an antibody against villin. Bottom lines were blotted with GAPDH antibody as a loading control. Representative images were selected. Densitometric analysis of the cytokeratine/GAPDH (G) and villin/GAPDH (H) bands from 2D organoids from CTR and CD. Columns represent the mean, and bars represent the standard deviations. The numbers of subjects analysed are indicated.

I. Western blot analysis of total protein lysates of 3D and 2D organoids from CTR and CD patients. Upper lines were blotted with an antibody against the phosphorylated form of NF-κB (pNF-κB). Lower lines were blotted with anti-GAPDH antibody as a loading control. Representative images were selected. L. Densitometric analysis of the pNF-κB/GAPDH bands from 3D and 2D organoids from CTR

and CD. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: * = p<0.05; ** = p<0.01. M. Western blot analysis of total protein lysates of 3D and 2D organoids from CTR and CD. Upper lines were blotted with an antibody against the phosphorylated form of ERK (pERK). Bottom lines were blotted with anti-ERK antibody as a loading control. Representative images were selected. N. Densitometric analysis of the pERK/ERK bands from CTR and CD. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: * = p<0.05; ** = p<0.01.

2.4 Organoids from CD patients were more sensitive to P31-43

CD cells responded to inflammatory stimuli such as the A-gliadin peptide P31-43 by increasing inflammatory markers expression [5]. To determine if there was a difference in sensitivity between CTR and CD organoids, we treated them with P31-43 at concentrations that did not affect CTR organoids (Figure 6).

P31-43 concentrations of 10 μg/ml and 20 μg/ml did not increase the expression of the inflammatory markers pNF-κB, pERK, IL-1β and IL-6 in control organoids (Figure 6). We analysed pNF-κB and pERK by WB and IL-1β and IL-6 by quantitative PCR and ELISA (Figure 6).

On the other hand, in CD organoids, the expression of the inflammatory markers pNF-κB and pERK increased when stimulated with P31-43 at 10 μg/ml (pNF-κB*p* value <0.05, pERK: *p* value <0.01) and 20 μg/ml (pERK: *p* value <0.05) (Figure 6 A-H).

As measured by quantitative PCR of the total mRNA, IL-1β and IL-6 (Figure 6 I-N) expression increased in CD organoids after both 10 μg/ml (IL1 beta: *p* value <0.0001, IL-6 *p* value <0.05) and 20 μg/ml (IL1 beta: *p* value <0.0001, IL-6 *p* value <0.001) P31-43.

ELISAs (Figure 6 O-R) on CD organoid culture media revealed that IL-1β expression was increased after 10 μg/ml P31-43 (*p* value <0.05), whereas IL-6 expression did not increase. These results indicate that CD enterocytes were more sensitive to the inflammatory stimuli of gliadin peptide P31-43 than control enterocytes.

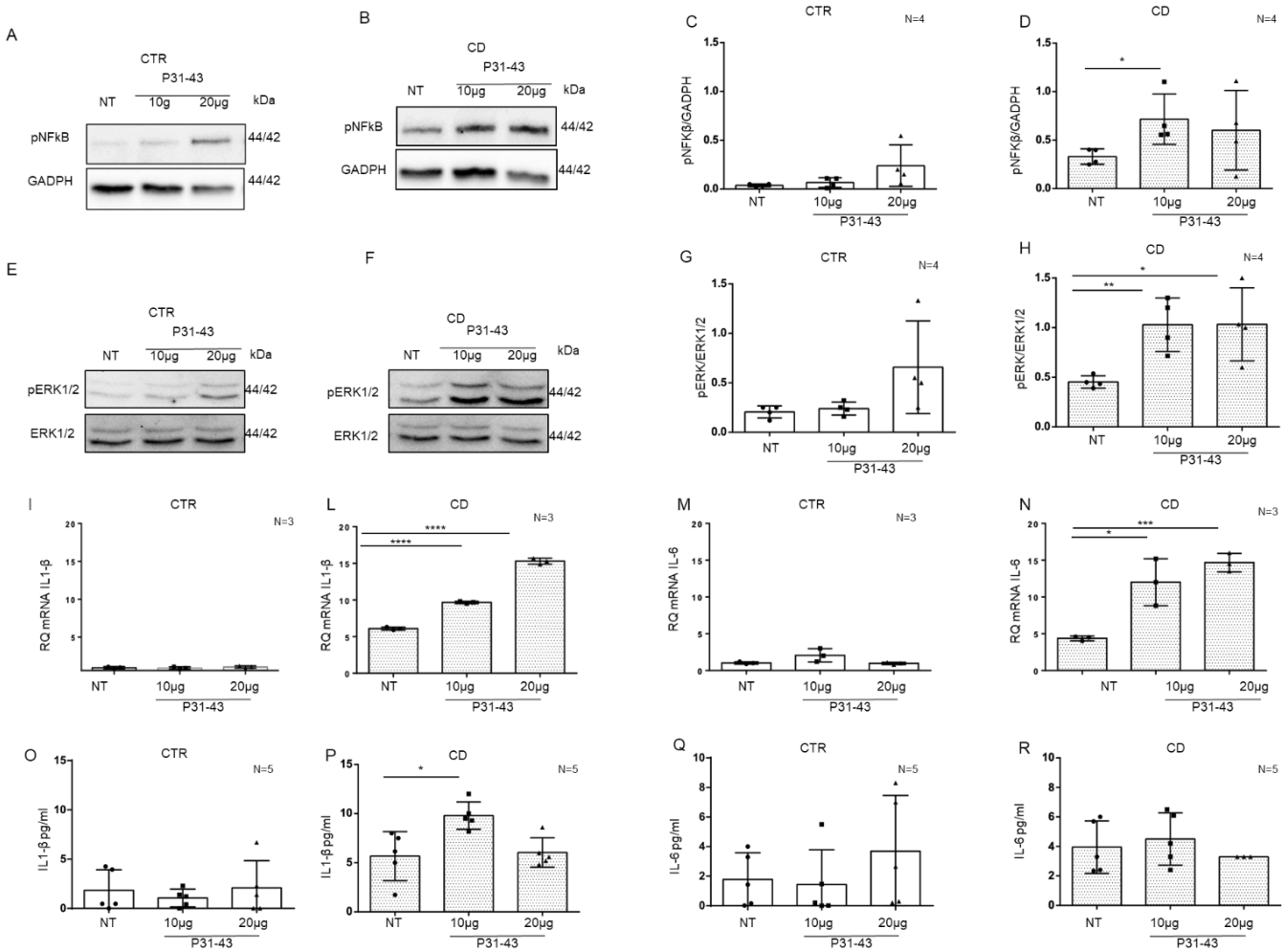

Figure 6. Organoids from CD patients, but not from CTR patients, are inflamed by P31-43. Western blot analysis of total protein lysates of organoids from CTR (A) and CD (B) before (NT) and after treatment with low concentrations of P31-43 (10 μg/ml (10 μg) and 20 μg/ml (20 μg)). Upper

lines were blotted with an antibody against the phosphorylated form of NF-κB (pNF-κB). Bottom lines were blotted with anti-GAPDH antibodies as a loading control. Representative images were selected. C and D Densitometric analysis of the pNF-κB/GAPDH bands from CTR and CD before and after treatment with P31-43 as indicated. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviations. Student's *t test*: * = $p<0.05$.E and F.Western blot analysis of total protein lysates of organoids from CTR (E) and CD (F) patients before (NT) and after treatment with P31-43 as indicated. Upper lines were blotted with an antibody against the phosphorylated form of ERK (pERK). Bottom lines were blotted with anti-ERK antibody as a loading control. Representative images were selected. G and H. Densitometric analysis of the pERK/ERK bands from CTR and CD before and after treatment with P31-43 as indicated. The numbers of subjects analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: * = $p<0.05$; ** = $p<0.01$.I-L. Quantitative PCR analysis of IL-1β mRNA levels in organoids from CD patients compared to CTR organoids. The numbers of subjects analysed are indicated. Student's *t test*: ****= $p<0.0001$.M-N. Quantitative PCR analysis of IL-6 mRNA levels in organoids from CD patients compared to CTR organoids. The numbers of organoids analysed are indicated. Student's *t test*: *=$p<0.05$; ***= $p<0.001$.

O-P. ELISA showing IL-1β protein levels in the culture media of CD organoids compared to CTR organoids before (NT) and after treatment with P31-43 as indicated. The numbers of organoids analysed are indicated. The columns represent the mean, and bars represent the standard deviation. Student's *t test*: * = $p<0.05$. Q and R. ELISA showing IL-6 protein levels in the culture media of CD and CTR

organoids before (NT) and after treatment with P31-43 as indicated. The numbers of organoids analysed are indicated. The columns represent the mean, and bars represent the standard deviation.

2.5 Organoids from CD patients were more sensitive to Lox

CD cells and biopsies responded to the inflammatory stimuli of Toll-like receptor 7 viral ligand, Lox, by increasing the levels of inflammatory markers [8,21]. To understand if there was a difference in sensitivity between CTR and CD organoids to viral ligand stimuli, we treated them with Lox at concentrations that did not affect CTR organoids (Figure 7).

In control organoids, 50 μM and 125 μM Lox (Figure 7) did not increase the expression of the inflammatory markers pNF-κB, pERK, IL-1β and IL-6, with the exception of pERK, which was significantly increased only by 125 μM Lox (p value <0.01). As before, we analysed pNF-κB and pERK by WB and IL-1β and IL-6 by quantitative PCR and ELISA (Figure 7).

On the other hand, in CD organoids, treatment with Lox increased the expression of the inflammatory markers pNF-κB and pERK at both 50 μM (pNF-κB, p value <0.05) and 125 μM (pERK, p value <0.05) concentrations, according to WB analysis (Figure 7 A-H).

Stimulation with 50 μM (p value <0.0001) and 125 μM (p value <0.01) Lox increased the levels of IL-6 mRNA (Figure 7 I-N) but not IL-1β mRNA.

ELISAs (Figure 7 O-R) on CD organoid culture media revealed that IL-1β was increased after 50 μM Lox (p value <0.05) treatment, whereas IL-6 was not increased. These results indicate that CD enterocytes were more sensitive than control enterocytes to inflammatory stimuli of different origins.

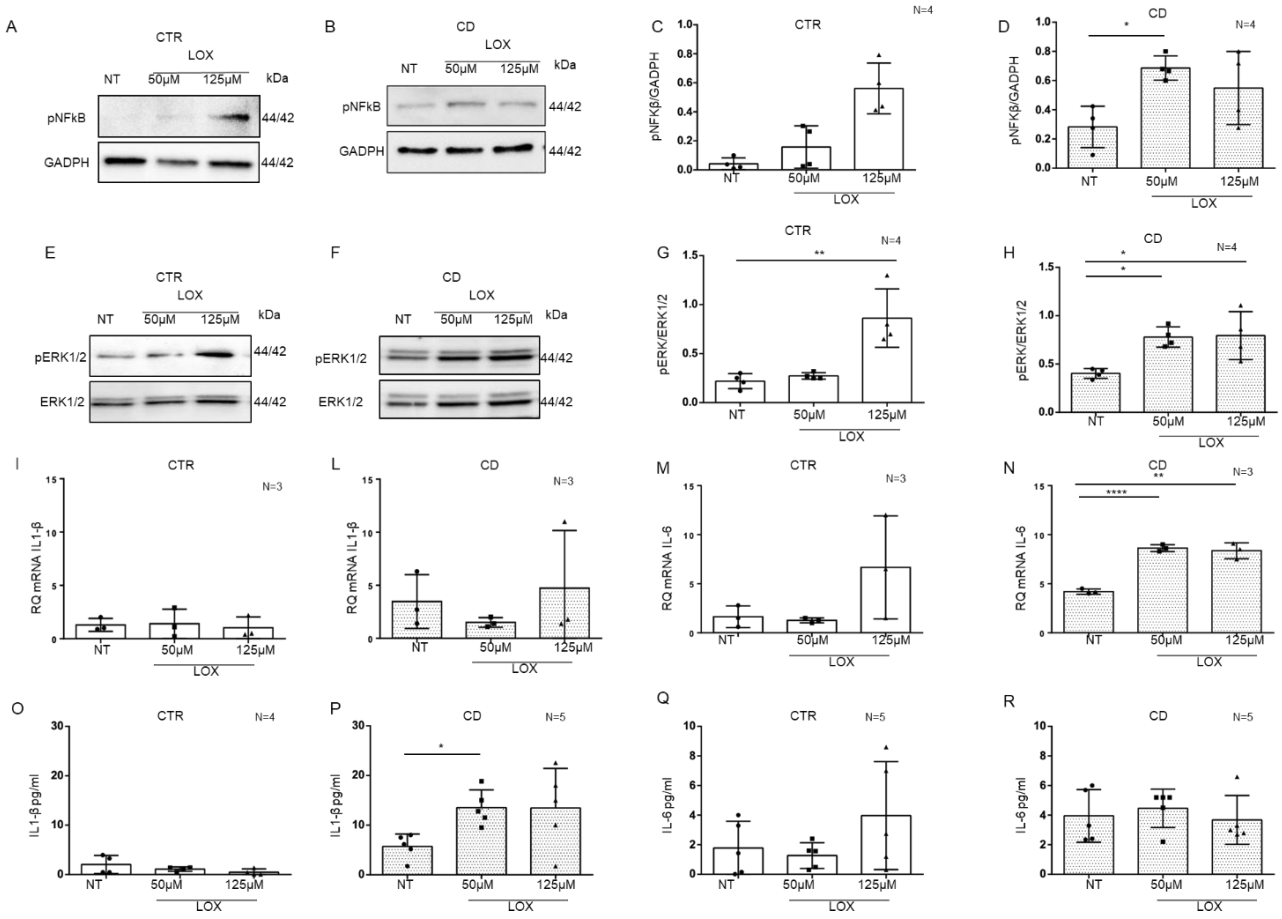

Figure 7. Organoids from CD patients, but not from CTR patients, are inflamed by Lox. Western blot analysis of total protein lysates of organoids from CTR (A) and CD (B) patients before (NT) and after treatment with low concentrations of Lox (50 μM and 125 μM). Upper lines were blotted with an antibody against the phosphorylated form of NF-κB (pNF-κB). Bottom lines were blotted with an- ti-GAPDH antibody as a loading control. Representative images were selected. C-D. Densitometric analysis of the pNF-κB/GAPDH bands from CTR and CD before and after treatment with Lox as indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: *= $p<0.05$.E and F.Western blot analysis of total protein lysates of organoids from CTR (E) and CD (F) patients before (NT) and after treatment with Lox as indicated. Upper lines were blotted with an antibody against the phosphorylated form of ERK (pERK). Bottom lines were blotted with anti-ERK an-

tibody as a loading control. Representative images were selected. G and H Densitometric analysis of the pERK/ERK bands from CTR and CD before and after treatment with Lox as indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: *= $p<0.05$; **= $p<0.01$**.** I-L. Quantitative PCR analysis of IL-1β mRNA levels in organoids from CD patients compared to those from CTR patients. The numbers of organoids analysed is indicated. Columns represent the mean, and bars represent the standard deviation. M-N. Quantitative PCR analysis of IL-6 mRNA levels in organoids from CD patients compared to those from CTR patients. The numbers of organoids analysed are indicated. Student's *t test*. **= $p<0.01$; ****= $p<0.0001$.

O-P. ELISA showing IL-1β protein levels in the culture media of CD organoids compared to CTR organoids before (NT) and after treatment with Lox as indicated. The numbers of subjects analysed are indicated. The columns represent the media, and bars represent the standard deviation. Student's *ttest*: *= $p<0.05$. Q-R. ELISA showing IL-6 protein levels in the culture media of CD and CTR organoids before (NT) and after treatment with Lox as indicated. The numbers of organoids analysed are indicated. The columns represent the media, and bars represent the standard deviation.

3. Discussion

The mucosa of the small intestine is the primary target of CD. Gluten peptides interact with the epithelium, cross the epithelial barrier and induce an adaptive immune response against gluten in individuals with MHC class II DQ2/8 haplotypes. Only a few individuals with these haplotypes will develop CD after exposure to gluten, indicating that other factors influence the initiation and maintenance of the disease. One of these factors could be the altered functionality of the innate epithelial response [19].

In this manuscript, we described epithelial inflammation in CD biopsies using IL-1β and IL-6 as markers in patients at the acute phase of the disease (GCD-CD), after remission of the intestinal lesion (GFD-CD), and patients with anti-TTG antibodies, who were genetically predisposed to CD (Pot-CD) yet had normal intestinal morphology for 2-3 years (on average) prior to the onset of the intestinal lesion. We derived organoids from intestinal biopsies of GCD-CD and CTR patients and tested them for markers of inflammation, such as pNF-κB, pERK, IL-1β and IL-6, and found that these markers were altered in CD organoids. In CD organoids the inflammation was persistent. Moreover, CD organoids were more sensitive than controls to inflammatory stimuli such as gliadin peptide P31-43 and the TLR7 receptor ligand Lox.

Intestinal inflammation in CD has been described by many different studies, in patients at both the GCD and GFD stages and before and after gluten challenge [8,10,11,13,16]. It is now clear that in CD, several different factors, such as cellular vulnerability, the proinflammatory effects of gluten and other wheat proteins, Western diet and other environmental triggers, such as viruses, converge to prepare, and/or amplify the TC-mediated response to gluten [5,8]

Moreover, changes at the mRNA and protein levels of the inflammasome pathway were found in intestinal epithelial cells purified from CD intestinal biopsies and analysed for gene expression. These results indicate that intestinal epithelial cells play a key role in small intestinal inflammation in CD [19]

The IL-1β and IL-6 cytokines are important mediators of the inflammatory response and are involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis[22,26].

In this manuscript, we found that IL-1β and IL-6 levels increased in CD enterocytes, not only in the acute phase of the disease but also in the remission phase and in potential patients before the onset of intestinal disease. Interestingly, in our experiments, both IL-1β and IL-6 levels were altered mainly in the epithelium in all stages of the disease. The presence of inflammation in the Pot-CD group is particularly interesting, as it indicates that inflammation of the epithelium precedes mucosal remodellings, and points to the intestinal epithelium as a key component of the inflammatory response in CD.

The possibility of growing small intestinal organoids has given many researchers a new tool to studythe role of the intestinal epithelium in several different diseases [24]. Organoids are a miniaturized and simplified version of an organ produced in vitro in three dimensions with realistic microanatomy. Intestinal organoids are derived from crypt stem cells. Several different groups have used organoids to study CD, revealing the presence of increased staminality, permeability, inflammasome activity, and innate immunity genes with respect to healthy individuals [19,27]. Extracellular matrix (ECM) genes were decreased in CD organoids compared to control individuals [25]. Taken together, these observations indicate that CD intestinal epithelial cells are constitutively different from those in healthy individuals.

In intestinal organoids from IBD, inflammation disappears after 1 week in culture and can be regained only upon INF-αtreatment [23]. Therefore, in IBD, inflammation in intestinal organoids is regarded as a residual effect of the tissue of origin. We derived organoids from the intestinal epithelium of CD patients, IBD patients and healthy individuals. We confirmed the data on IBD organoids available in the literature. In our experiments, IBD (both Crohn's and ulcerative colitis) organoids were no longer inflamed after 4 weeks in culture, and both pNF-κB and pERK levels were not different from the control organoids.

In contrast, in CD organoids, we found increased markers of inflammation, such as pNF-κB, pERK, IL-1β and IL-6, at the protein and mRNA levels. In contrast to that in IBD, inflammation in CD organoids was persistent, as the levels of pNF-κB and pERK did not decrease after more than ten weeks in culture. This suggests that the inflammation in CD organoids is not a residual effect of the tissue of origin but is constitutive.

Possible constitutive alterations in CD, which appear to be independent of the stage of the disease and the gluten content in the diet, have been recently described in vivo in the literature [11,28,29]. Interestingly, several cytokines related to the inflammatory pathway were increased in at-risk CD infants before the onset of the disease and the introduction of gluten to the diet (personal communication).

Moreover, in CD biopsies and fibroblasts, increased sensitivity to inflammatory triggers such as gliadin peptide P31-43, IL-15 and Toll-7-specific viral ligand Lox have been described [17,21,30,31]

For this reason, we treated CD and CTR organoids with two different environmental inflammatory stimuli, gliadin peptide P31-43 and Lox. P31-43 and Lox, ineffective in CTR organoids, were able to induce the activation of NF-κB and ERK and increase IL-1β and IL-6 levels in CD organoids. In CD

biopsies and in intestinal organoids, increased sensitivity to inflammatory stimuli from bacteria has been described [19], indicating that intestinal organoids from CD patients are more sensitive to proinflammatory stimuli.

In conclusion, the factors that create a proinflammatory environment in the CD intestine can be exogenous, such as food and viruses but can also be endogenous (Figure 8). In fact, low-grade inflammation of the CD epithelium, probably constitutive, is present even before intestinal damage. Intestinal organoids reproduced this constitutive inflammation and thus represent a good model for studying epithelial inflammation in CD. Moreover, the intestinal epithelium in CD is more sensitive to proinflammatory stimuli, including gliadin and viruses. Taken together, these observations point to constitutive alterations, probably genetic or epigenetic, that render the CD epithelium more sensitive to inflammatory stimuli such as food components, viruses and microbiota (Figure 8).

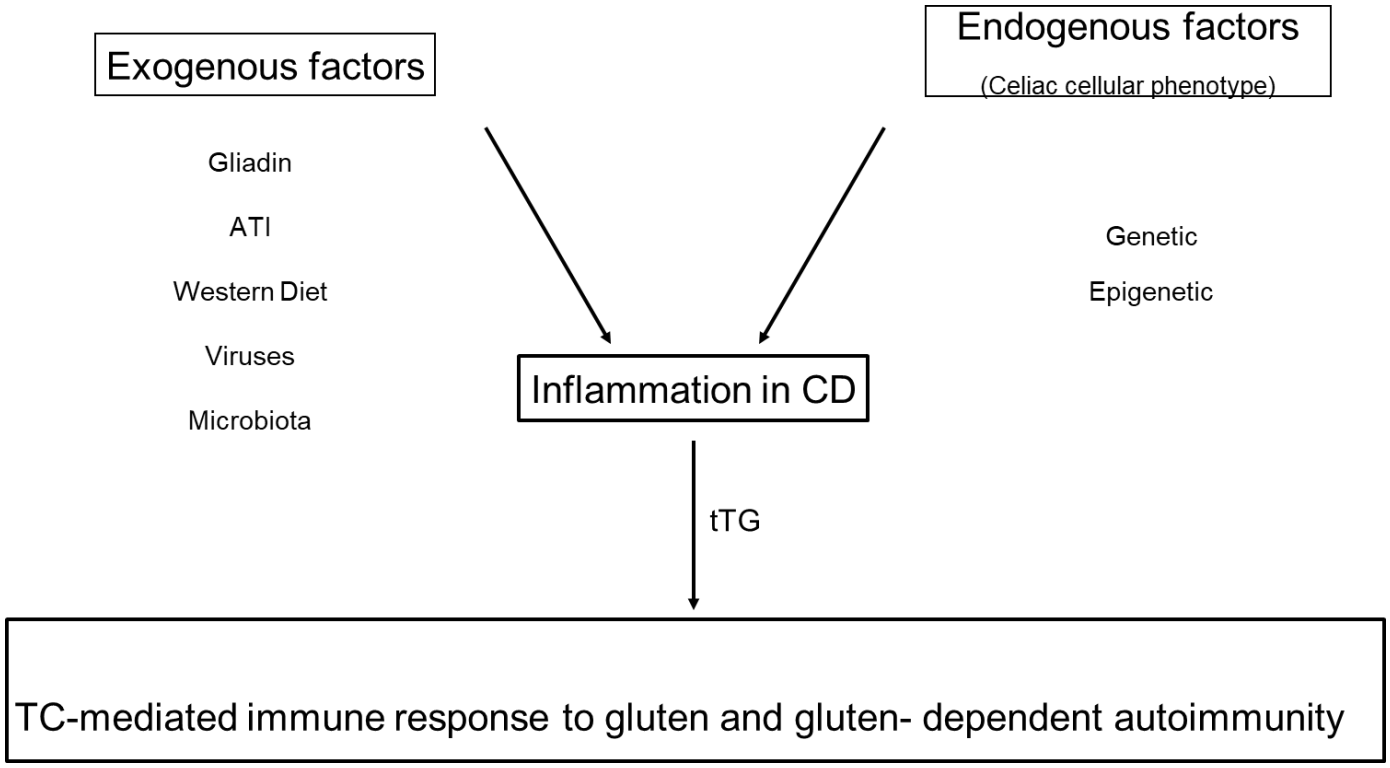

Figure 8:Inflammation in the CD epithelium is mediated by exogenous and endogenous factors. Inflammation in CD is induced and maintained by several different factors, both exogenous and endogenous, probably generated by a specific genetic/epigenetic background. Exogenous factors, in addition to gliadin, include ATIs (amylase trypsin inhibitors), other food components of the Western diet, viruses and microbiota. Endogenous factors render cells more sensitive to proinflammatory stimuli from the environment. The inflamed environment allows the activation of the T cell mediated response to gliadin, modified by tissue transglutaminase (tTG).

4. Materials and Methods

Table 1. Patient characteristics.

Patients	Range Age (Years)	Sex	Biopsy (Marsh classification*)	Serum Anti-TG2 (U/ml)	Antiendomysial Antibody (EMA)
Controls (N=8)	10-18	M=4, F=4	8=T0	0-1.5	Negative
GCD-CD (N=8)	3-15	M=3, F=5	10=T3c 2=T3cb	>50	Positive
GFD-CD (N=5)	5-18	F=5	3=T0 2=T1	0-1.5	Negative
Pot-CD (N=4)	7-12	M=2, F=2	3=M0 1=T1	9.3-57	3=positive 1=ND

T0: Normal; T1: infiltrative lesion; T3: Flat destructive lesion (a: mild, b: moderate, c: total)

4.1 Organoids

One to two duodenal biopsies per individual were taken with standard endoscopic ERGDS during routine gastroduodenoscopy and placed in ice-cold 10 ml isolation buffer (5.6 mmol/L Na_2HPO_4 (Sigma S7907; Sigma–Aldrich, St Louis, MO), 8.0 mmol/L KH_2PO_4 (Sigma P5655; Sigma–Aldrich), 96.2 mmol/L NaCl (Sigma S5886; Sigma–Aldrich), 1.6 mmol/L KCl (Sigma P5405; Sigma–Aldrich), 43.4 mmol/L sucrose (Fisher BP220-1; Thermo Fisher Scientific, Waltham, MA), and 54.9 mmol/L D-sorbitol (Fisher BP439-500; Thermo Fisher Scientific)) in deionized water. Crypt units were isolat- ed according to the protocol of Yuli Wang et al. [32] with minor variations. Briefly, after 60 minutes, the biopsy samples were further enzymatically digested with collagenase (2 mg/ml, Sigma–Aldrich Milan, Italy) in washing buffer (WB) containing penicillin (100 units ml^{-1}), streptomycin (0.1 mg ml^{-1}), l-glutamine (2 mM) and FBS (10%, vol/vol) in DMEM/F12 with HEPES on ice for 30 minutes. The digest was filtered through a 70 µm strainer (Falcon, Germany) and the strainer was rinsed with an additional 10 ml of WB. Crypts were collected by centrifugation at 500×g for 5 min. The supernatant was discarded, the crypts were carefully resuspended in 40 µl of ice-cold Matrigel matrix (Corning 35623 Milan, Italy) to enable three-dimensional growth in 48-well plates; the plates were incubated in a cell culture incubator at 37 °C and 5% carbon dioxide for 10 minutes to allow the Matrigel to solidify. Afterwards, 300 µl cell culture medium enriched with supplements (CM-S) was added to each well and was replaced every second day. The organoids were used for assays or cryopreserved at −150 °C. To cryopreserve organoids, they were washed with ice-cold PBS EDTA to remove Matrigel and collected by centrifugation at 500×g for 5 min. Organoid pellets were suspended in 1ml WB, 10% faecal calf serum (FCS, Biochrom, Germany), and 10% dimethyl sulfoxide, slowly frozen to −80 °C in a

cryo freezing container (Nalgene, Germany), and then transferred to −150 °C for long-term storage. For further research, the cryopreserved organoids were quickly thawed at 37 °C, transferred to 10 ml WB, centrifuged at 2000×g for 5 min, plated with Matrigel and cultured in CM-S medium. For 2D organoids, organoids were openly seeded in six wells pretreated with Matrigel diluted 1:40 in phosphate-buffered saline (PBS)

4.2 Culture medium to maintain organoids (CM-S).

Mouse L-cells that expressed Wnt3a, R-spondin, and noggin were commercially purchased (ATCC CRL-3276, Bio Tech Standards, Germany), and a conditioned medium (L-WRN) was prepared according tothe instructions and protocol of the manufacturer. Culture medium with supplements (CM-S) was prepared using 50% conditioned L-WRN medium and 50% fresh primary culture media: Advanced DMEM/F-12 [Invitrogen] 1 mM N-acetyl-L-cysteine (Sigma, Germany), 1x N-2 supplements (Gibco, Germany), 1 × B-27® supplements (Gibco, Germany), 50 ng/ml epidermal growth factor, 10 mM nicotinamide (Sigma, Germany), 10 nM Leu15-gastrin I (Sigma, Germany), 500 nM A8301 (inhibitor for ALK4/5/7; Sigma, Germany), 10 µM SB202190 (p38 MAP kinase inhibitor; Sigma, Germany), and 10 µM Y-27632 (p160 ROCK inhibitor; Tocris, Germany) in accordance with the protocols of Sato et al. [33], VanDussen et al. [34] and Yuli Wang et al. [32]. The organoids were cultured with 300 µl culture medium, which was changed every second or third day. After seven days, when the organoids had formed large circular structures, they were isolated from the Matrigel matrix and split. For each passage, the spheroid/Matrigel was scratched into 500 µL of PBS containing 0.5 mM EDTA (PBS-EDTA). The spheroids were pelleted by centrifugation at 500 g for 5 min, and the super-

natant was discarded. The spheroids were dissociated by incubation in 200 µL of 0.25% trypsin in PBS-EDTA for 60-90 sec. An additional 5 mL of WB was then added to inactivate the trypsin. The spheroids were pelleted by centrifugation at 500 g for 5 min. The supernatant was carefully removed, and the pellet was resuspended in Matrigel.

4.3 Fixing of organoids, OCT embedding and cryosectioning

After removing the organoid culture media, the human spheroids were washed from each well of a 6-well plate with 5 ml of 1X PBS at room temperature. The structures were fixed with 5 ml of 2% paraformaldehyde (PFA) and 0.1% glutaraldehyde (GA) in 1X PBS for 30 minutes at room temperature. After washing extensively with 5 ml of 1X PBS to remove the fixing solution, the organoid domes were carefully removed with a scoop or spatula and placed in a 50 ml conical tube containing 20% sucrose in 1X PBS.The tube was left at 4 °C overnight or for three days, until the domes fell to the bottom of the tube. The domes were removed from the sucrose solution and placed in a mould containing optimal cutting temperature (OCT) compound. Several domes were placed on each mould; they were snap frozen and stored at -80 °C. Using a cryotome, we cut the organoid block into cryosections approximately 10 µm thick.

4.4 Immunostaining

After washing the slides with 1X PBS to remove OCT for 3D organoids, the 2D organoids were openly seeding in six wells pretreated with Matrigel diluted 1:40 in PBS; the tissues were permeated with 0.15% Triton/1X PBS for 15 minutes at room temperature. The cells were washed 3 times with 1X

PBS for 10 minutes each time. The slides were blocked with 3% BSA/1X PBS (blocking solution) or 10% FBS/1X PBS for 1 hour at room temperature. The primary antibody anti-rabbit anti-cytokeratin (DakoCarpinteria, Ca USA) was diluted in blocking solution overnight at 4 °C in a humidified chamber. Then the cells were washed 3 times with 1X PBS for 10 minutes each time.Alexa Fluor 546 donkey anti-rabbit secondary antibody (Invitrogen, Milan, Italy) was added to the organoids. The cells were then washed 3 times with 1X PBS for 10 minutes each time, mounted with Mowiol (Sig- ma–Aldrich, Milan, Italy) and observed under a Zeiss LSM 510 confocal microscope (Germany) [17].

4.5 Western blot

The human spheroids were seeded in six multiwells (Corning, Milan, Italy) coated with Matrigel diluted 1:40 in phosphate buffered saline (PBS) for 3 days. After they were stimulated with P31–43 or LOX for various times at 37 °C, the organoids were homogenized in tissue homogenization buffer (50 mM Tris–HCl [pH 8], 150 mM NaCl, 5 mM MgCl2, 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM VO4, aprotinin, and LAP; all purchased from Sigma, Milan, Italy, except for LAP, which was purchased from Roche, Milan, Italy). The cell lysates were analysed using SDS–PAGE with a standard running buffer (25 mM Trizma, 192 mM glycine, and 0.1% SDS) and were transferred onto nitrocellulose membranes using Transbolt Turbo (BioRad Milan, Italy). The membranes were blocked with 5% nonfat dry milk and probed with rabbit anti-ERK1/2 and rabbit anti-pY-ERK1/2, (Elabscience, Milan, Italy) mouse anti-GAPDH, (Sigma–Aldrich, Milan, Italy), and rabbit anti-pY-NF-κB, (Cell Signalling, Euroclene, Milan, Italy). The bands were visualized using ECL (GE Healthcare, Amersham, Buckinghamshire, UK) with exposure times of 2–10 min. The band

intensity was evaluated by integrating all the pixels of the band after subtraction of the background to calculate the average of the pixels surrounding the band[17].

4.6 ELISA

The levels of IL-1β and IL-6 were measured using commercial test kits (Diaclone, Besancon Cedex, France) on cell culture supernatants.

4.7 RNAscope to detect IL-1β and IL-6 mRNA

IL-1β and IL-6 mRNA levels were detected by RNAscope™ 2.5 HD Assay – RED (Advanced Cell Diagnostics, Inc. Canada). Sample preparation, probe hybridization and signal detection were carried out according to the kit instructions. Positive signals were indicated by dot-shaped red granules in the crypts. Positive and negative probes were used as positive and negative controls. The red-stained cells were counted. Positive staining in more than 10% of the cells was considered positive, while less than 10% or colourless staining was defined as negative.

Briefly, biopsies included in paraffin were cut at 5 μm, slides were deparaffinized with xlylene twice for 5 minutes and 100% alcohol twice for 1 minute. Deparaffinized slides were incubated with hydrogen peroxide for 10 minutes at room temperature, submerged in the target retrieval reagent for 30 minutes at 99°, transferred to 100% alcohol for 3 minutes and dried at room temperature overnight.Each slide was incubated with protease plus for 30 minutes at 40° and then washed 3 times with distilled water. Slides were incubated with the appropriate probe for 2 hours at 40°, washed with wash buffer and incubated with hybridized Amp (from Amp 1 to Amp 6) according to the protocol. To de-

tect the signal, slides were incubated with fast red solution, submerged in staining dishes containing 50% haematoxylin and mounted with EcoMount.

4.8 PCR

Total RNA was extracted from organoids using a RNeasy Mini Kit (Qiagen, Milan, Italy). The mRNA concentration was measured using a Nanodrop spectrophotometer, and the RNA quality was analysed using agarose gel electrophoresis in Tris/Borate/ethylenediaminetetraacetic acid (EDTA) buffer (TBE, Sigma, Milan, Italy). RNA (1 µg) was reverse transcribed into cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Milan, Italy) according to the manufacturer's protocol. The experiments were performed with approximately 40 ng of cDNA templates, according to the manufacturer's protocol (TaqMan Gene Expression Assay), using a 7900 HT Fast Real-Time PCR system. The gene expression assay used to detect the IL-1β gene was Hs01555410_m1 (Applied Biosystems, Thermo Fisher Scientific Inc., Monza, Italy), and the probe was located on Chr.2: 112829758-112836842 on Build GRCh38; for the IL-6 gene Hs00174131_m1 (Applied Biosystems, Thermo Fisher Scientific Inc., Monza, Italy) was used, and the probe was located on Chr.7: 22725889-22732002 on Build GRCh38.The expression of each gene was normalized to the expression of an endogenous housekeeping gene (HPRT1). Relative quantification was performed using the ΔΔCt method. SDS software (ABI, version 1.4 or 2.4) was used to analyse the raw data.

4.9 Statical analysis

GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) was used for statistical analyses and to construct graphical representations. Statistical analyses of the differences included Student's *t tests*. A p value < 0.05 was considered statistically significant. Two-tailed comparisons were used for all statistical analyses. The sample size was chosen after considering the variance of the control samples, and the number of samples needed to assess the extent of the expected effect was estimated to be 3 or 4. Therefore, the chosen sample size was 3, 4, or (more often) 5.

4.5 Ethical Statement: The Ethical Committee of the Federico II University of Naples approved the protocol of this study (ethical approval: 115/09/ESPROT). Written informed consent was obtained from all patients or from next of kin, caretakers, or guardians on behalf of the minors /child participants involved in our study. The authors confirm that all methods were performed in accordance with the relevant guidelines and regulations.

Supplementary Materials: The following supporting information can be downloaded at:

Author Contributions: M.P, performed the mRNA in situ experiments and contributed data analysis; M.C, G.L, C.B performend WB experiments and RT PCR; L.R. prepared the immunostaining slides and cared for the biobank; A.R and R.T. led the clinical recruitment of patients; A.S. contributed to study conception and writing the manuscript; MV.B. led the study conception, design, performance, and wrote the text. M.N led the study conception and design and performed experiments.

Funding: The authors declare no conflict of interest.

Informed Consent Statement: The Ethical Committee of the Federico II University of Naples approved the protocol of this study (ethical approval: 115/09/ESPROT). Written informed consent was obtained from all patients or from next of kin, caretakers, or guardians on behalf of the minors /child participants involved in our study. The authors confirm that all methods were performed in accordance with the relevant guidelines and regulations.

Conflicts of Interest: "The authors declare no conflict of interest."

5. References

1. Sollid LM, Jabri B. Triggers and drivers of autoimmunity: lessons from coeliac disease. *Nat Rev Immunol.* Apr 2013;13(4):294-302.

2. Lindfors K, Ciacci C, Kurppa K, et al. Coeliac disease. *Nat Rev Dis Primers.* Jan 10 2019;5(1):3.

3. Bouziat R, Hinterleitner R, Brown JJ, et al. Reovirus infection triggers inflammatory responses to dietary antigens and development of celiac disease. *Science.* Apr 7 2017;356(6333):44-50.

4. Barone MV, Troncone R, Auricchio S. Gliadin peptides as triggers of the proliferative and stress/innate immune response of the celiac small intestinal mucosa. *Int J Mol Sci.* Nov 7 2014;15(11):20518-20537.

5. Chirdo FG, Auricchio S, Troncone R, Barone MV. The gliadin p31-43 peptide: Inducer of multiple proinflammatory effects. *Int Rev Cell Mol Biol.* 2021;358:165-205.

6. Junker Y, Zeissig S, Kim SJ, et al. Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4. *J Exp Med.* Dec 17 2012;209(13):2395-2408.

7. Barroso M, Beth SA, Voortman T, et al. Dietary Patterns After the Weaning and Lactation Period Are Associated With Celiac Disease Autoimmunity in Children. *Gastroenterology.* Jun 2018;154(8):2087-2096.e2087.

8. Barone MV, Auricchio S. A Cumulative Effect of Food and Viruses to Trigger Celiac Disease (CD): A Commentary on the Recent Literature. *Int J Mol Sci.* Feb 18 2021;22(4).

9. Deets KA, Vance RE. Inflammasomes and adaptive immune responses. *Nat Immunol.* Apr 2021;22(4):412-422.

10. Stamnaes J, Stray D, Stensland M, et al. In Well-Treated Celiac Patients Low-Level Mucosal Inflammation Predicts Response to 14-day Gluten Challenge. *Adv Sci (Weinh).* Feb 2021;8(4):2003526.

11. Dotsenko V, Oittinen M, Taavela J, et al. Genome-Wide Transcriptomic Analysis of Intestinal Mucosa in Celiac Disease Patients on a Gluten-Free Diet and Postgluten Challenge. *Cell Mol Gastroenterol Hepatol.* 2021;11(1):13-32.

12. Hayden MS, Ghosh S. NF-κB in immunobiology. *Cell Res.* Feb 2011;21(2):223-244.
13. Trynka G, Zhernakova A, Romanos J, et al. Coeliac disease-associated risk variants in TNFAIP3 and REL implicate altered NF-kappaB signalling. *Gut.* Aug 2009;58(8):1078-1083.
14. Fernandez-Jimenez N, Castellanos-Rubio A, Plaza-Izurieta L, et al. Coregulation and modulation of NFκB-related genes in celiac disease: uncovered aspects of gut mucosal inflammation. *Hum Mol Genet.* Mar 1 2014;23(5):1298-1310.
15. Castellanos-Rubio A, Bilbao JR. Profiling Celiac Disease-Related Transcriptional Changes. *Int Rev Cell Mol Biol.* 2018;336:149-174.
16. Castellanos-Rubio A, Ghosh S. Disease-Associated SNPs in Inflammation-Related lncRNAs. *Front Immunol.* 2019;10:420.
17. Lania G, Nanayakkara M, Maglio M, et al. Constitutive alterations in vesicular trafficking increase the sensitivity of cells from celiac disease patients to gliadin. *Commun Biol.* 2019;2:190.
18. Barone MV, Zanzi D, Maglio M, et al. Gliadin-mediated proliferation and innate immune activation in celiac disease are due to alterations in vesicular trafficking. *PLoS One.* Feb 25 2011;6(2):e17039.
19. Pietz G, De R, Hedberg M, et al. Immunopathology of childhood celiac disease-Key role of intestinal epithelial cells. *PLoS One.* 2017;12(9):e0185025.
20. Barone MV, Zimmer KP. Endocytosis and transcytosis of gliadin peptides. *Mol Cell Pediatr.* Dec 2016;3(1):8.
21. Nanayakkara M, Lania G, Maglio M, et al. P31-43, an undigested gliadin peptide, mimics and enhances the innate immune response to viruses and interferes with endocytic trafficking: a role in celiac disease. *Sci Rep.* Jul 17 2018;8(1):10821.
22. Rose-John S. Interleukin-6 Family Cytokines. *Cold Spring Harb Perspect Biol.* Feb 1 2018;10(2).
23. Arnauts K, Verstockt B, Ramalho AS, Vermeire S, Verfaillie C, Ferrante M. Ex Vivo Mimicking of Inflammation in Organoids Derived From Patients With Ulcerative Colitis. *Gastroenterology.* Oct 2020;159(4):1564-1567.
24. Li VSW. Modelling intestinal inflammation and infection using 'mini-gut' organoids. *Nat Rev Gastroenterol Hepatol.* Feb 2021;18(2):89-90.
25. Dieterich W, Neurath MF, Zopf Y. Intestinal ex vivo organoid culture reveals altered programmed crypt stem cells in patients with celiac disease. *Sci Rep.* Feb 26 2020;10(1):3535.
26. Amaral GA, Alves JD, Honorio-França AC, et al. Interleukin 1-beta is Linked to Chronic Low-Grade Inflammation and Cardiovascular Risk Factors in Overweight Adolescents. *Endocr Metab Immune Disord Drug Targets.* 2020;20(6):887-894.
27. Freire R, Ingano L, Serena G, et al. Human gut derived-organoids provide model to study gluten response and effects of microbiota-derived molecules in celiac disease. *Sci Rep.* May 7 2019;9(1):7029.
28. Galatola M, Izzo V, Cielo D, et al. Gene expression profile of peripheral blood monocytes: a step towards the molecular diagnosis of celiac disease? *PLoS One.* 2013;8(9):e74747.
29. Auricchio R, Galatola M, Cielo D, et al. A Phospholipid Profile at 4 Months Predicts the Onset of Celiac Disease in at-Risk Infants. *Sci Rep.* Oct 4 2019;9(1):14303.
30. Auricchio R, Stellato P, Bruzzese D, et al. Growth rate of coeliac children is compromised before the onset of the disease. *Arch Dis Child.* Oct 2020;105(10):964-968.

31. Bernardo D, Garrote JA, Allegretti Y, et al. Higher constitutive IL15R alpha expression and lower IL-15 response threshold in coeliac disease patients. *Clin Exp Immunol.* Oct 2008;154(1):64-73.

32. Wang Y, DiSalvo M, Gunasekara DB, et al. Self-renewing Monolayer of Primary Colonic or Rectal Epithelial Cells. *Cell Mol Gastroenterol Hepatol.* Jul 2017;4(1):165-182.e167.

33. Sato T, Vries RG, Snippert HJ, et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature.* May 14 2009;459(7244):262-265.

34. VanDussen KL, Marinshaw JM, Shaikh N, et al. Development of an enhanced human gastrointestinal epithelial culture system to facilitate patient-based assays. *Gut.* Jun 2015;64(6):911-920.

International Journal of *Molecular Sciences*

MDPI

Type of the Paper (Article, Review, Communication, etc.)

Gliadin peptide P31-43 induces mTor/NFkB activation and reduces autophagy: role of Lactobacillus Paracasei CBA L74 postbiotc.

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Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *Int. J. Mol. Sci.* **2022**, *23*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Firstname Lastname

Received: date
Accepted: date
Published: date

Abstract:

Celiac disease (CD) is an autoimmune disease characterized by an altered immune response stimulated by gliadin peptides that are not digested and cause damage to the intestinal mucosa.

The aim of this study was to investigate whether the postbiotic of Lactobacillus Paracasei (LP) could prevent the action of gliadin peptides on mTOR, autophagy and inflammatory response.

Most of the experiments performed were conducted on intestinal epithelial cells CaCo-2 treated with peptic-tryptic digest of gliadin (PTG) and with P31-43. Furthermore, we pretreated the CaCo-2 with the postbiotic of LP before treatment with the previous described stimuli. In both cases, we evaluated the levels of pmTOR, p70S6k, p4EBP-1 for the mTOR pathway, pNFkB and pERK for inflammation and LC 3 and p62 for autophagy. For autophagy, we also used immunoflorescence analysis. Using intestinal organoids derivated from celiac (CD) patients we have analyzed effect of gliadin after postbiotic pretreatment of LP on inflammation marker NFkB. With these experiments, we have shown that gliadin peptides are able to induce the increase of the inflammatory response in a more complex model of intestinal epithelial cells and that pretreatment with the postbiotic of LP is able to prevent these effects. In conclusion, postbiotic pretreatment of LP could be considered for in vivo clinical trials.

Keywords: Celiac disease, post-biotic, oganoids, inflammation , mTor, autophagy.

1. Introduction

The gut is the largest immune organ in the body [1]. Approximately 100 trillion bacteria are associated with our gastrointestinal tract. The microbiota is considered a "super-organism" and is an integral part of the gastrointestinal tract [2].

Numerous functions are attributed to the microbiota in the human gut; in fact, it competes (for space and nutrients) with potential pathogens, induces secretion of antimicrobial peptides, and stimulates differentiation and proliferation through interaction with intestinal epithelial cells [3, 4]which in turn regulate intestinal homeostasis [5-7]. In addition, they modulate the inflammatory response induced by nutrient excess as nutrients are modulators of various cellular functions and may be involved in tissue immune response and inflammation.

Celiac disease (CD) is a systemic immune-mediated disease caused by gluten and related prolamins contained in wheat, barley and rye. It occurs in genetically predisposed individuals and is characterized by a variable combination of gluten-dependent clinical manifestations, presence of specific autoantibodies in serum, positivity for HLA DQ2 and/or DQ8 haplotypes and enteropathy. To date, the gluten-free diet represents the only therapeutic option for these patients, who must eliminate gluten-containing grains from their diet for life.

Microbial dysbiosis has been largely reported in CD patients [8, 9]. Activation of innate immunity by gliadin peptides is an important component of the early events of the disease. In particular, the ''toxic'' A-gliadin peptide P31-43 induces several pleiotropic effects including Epidermal Growth Factor Receptor (EGFR)-dependent actin remodelling and proliferation in cultured cell lines and in enterocytes from CD patients and inflammation. [10, 11]

The mechanistic target of rapamycin (mTOR) network is an evolutionary conserved signaling hub that senses and integrates environmental and intracellular nutrient and growth factor signals to coordinate basic cellular and organismal responses such as cell growth, proliferation, apoptosis, and inflammation [12]. Gliadin sustains mTOR inflammatory response in celiac disease[13]. More over gliadin-derived peptides are able to stimulate enterocytes[14, 15] causing the up-regulation of pro-inflammatory cytokine expression such as the activation of the nuclear transcription factork-B (NF-kB) pathway in the small intestinal mucosa of CD patients [16].

Autophagy is crucial for cytoplasmic recycling, fundamental homeostasis, and cell survival[17, 18] and it is also an essential component of immune defense against bacterial pathogens such as Mycobacterium tuberculosis, Salmonella enteric, and Escherichia coli [19-21]. Thus, triggering autophagy is essential for cell survival during pathogen infection [22, 23]. The induction of autophagy involves numerous proteins and multiple signaling pathways. Microtubule-associated light chain 3 protein 1 (LC3, a mammalian homolog of yeast Atg8), a vital component of the elongation phase, is considered one of the marker of autophagy [24]. p62 (also known as SQSTM1), is a marker of autophagic flux, is selectively incorporated into autophagosomes and efficiently degraded by autophagy [25].

A recent interest is given by the use of prebiotics, probiotics and postbiotics to modify the intestinal microbiome and the major interest is focused mainly on the use of postbiotics, substance released by or produced through the metabolic activity of the microorganism, which exerts a beneficial effect on the host, either directly or indirectly[26].

In this paper, we analyzed the effect of the gliadin peptide P31-43 on the mTOR and autophagy pathways and the ability of the LP CBA L74 (LP) postbiotic to prevent these effects in CaCo-2 cells, an intestinal epithelial cell line, and organoids from intestinal biopsies of CD patients.

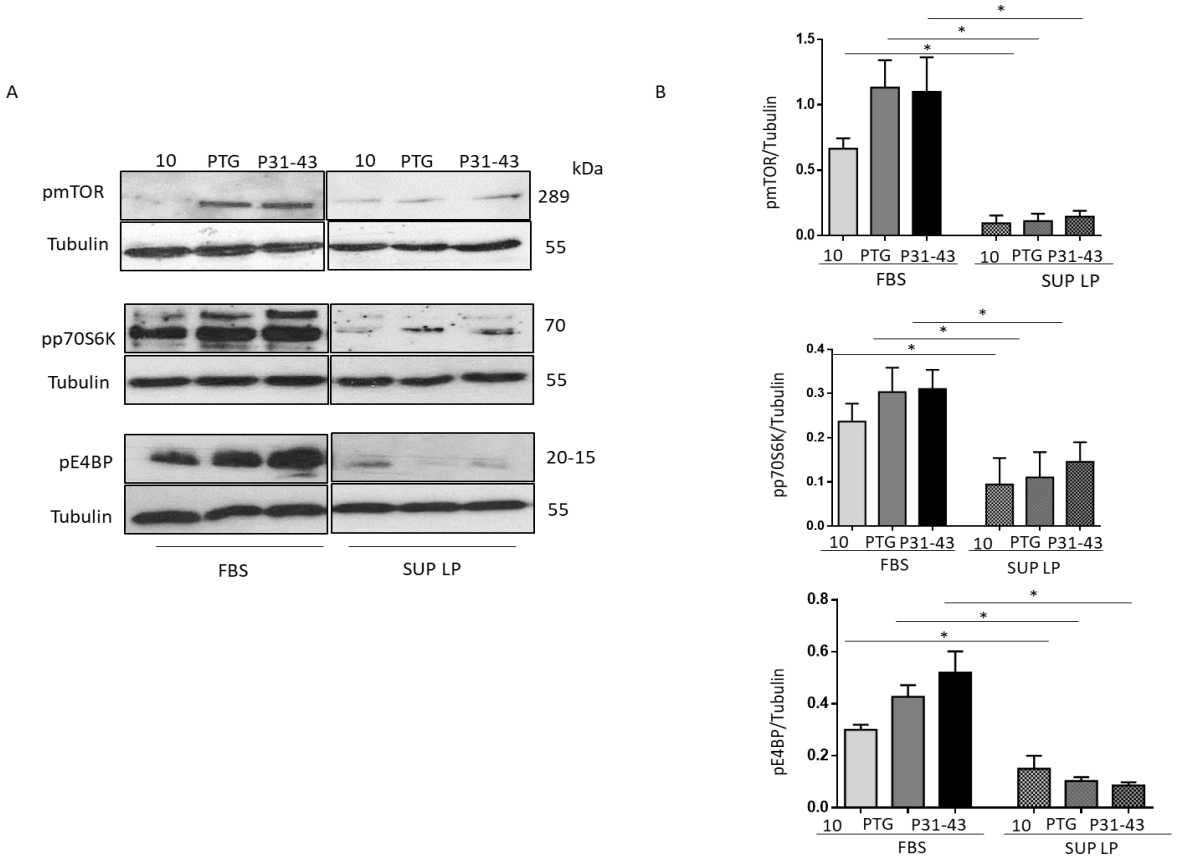
2. Results

Gliadin peptides were able to induce mTOR, p70S6 Kinase and 4EBP phosphorylation

The mammalian/mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that integrates a multitude of extracellular signals and intracellular cues to drive growth and proliferation.

We investigated the effects of the gliadin peptide P31–43 and PTG (peptic-tryptic digest of gliadin) on the mTOR and autophagy pathway in CaCo-2 cells, an intestinal epithelial cell line that is responsive to gliadin. To determine whether mTOR pathway is active in CaCo-2 cells we analyzed before and after treatment with PTG and p31-43 the phosphorylated/active form of mTOR and downstream targets p70S6 Kinase 1 (S6K1) and eIF4E Binding Protein (4EBP), by Western blot.

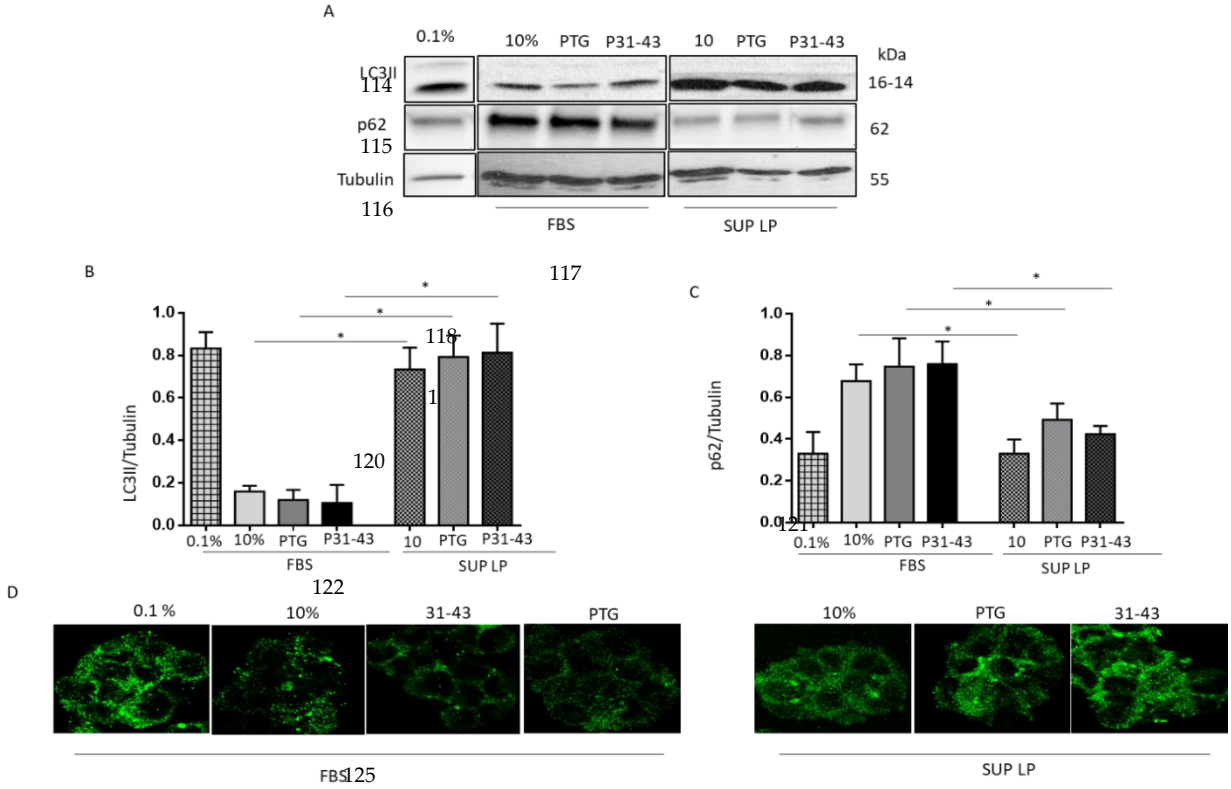
p-mTOR, p-S6K1 and p-4EBP were increased respect to untreated cells (NT) after treatment with both p31-43 and PTG. Pretreatment with LP postbiotic was able to prevent activation of the mTOR pathway in CaCo-2 cells. (Figure.1)



Gliadin peptides reduced the levels of LC3 and increased the levels p62 autophagy markers

In presence of nutrients restriction mTOR is generally dephosphorylated and authophagy is induced. The opposite is true in presence of nutrients. For these reasons we evaluated the authophagy pathway in CaCo-2 cells after treatment with P31-43 and PTG.

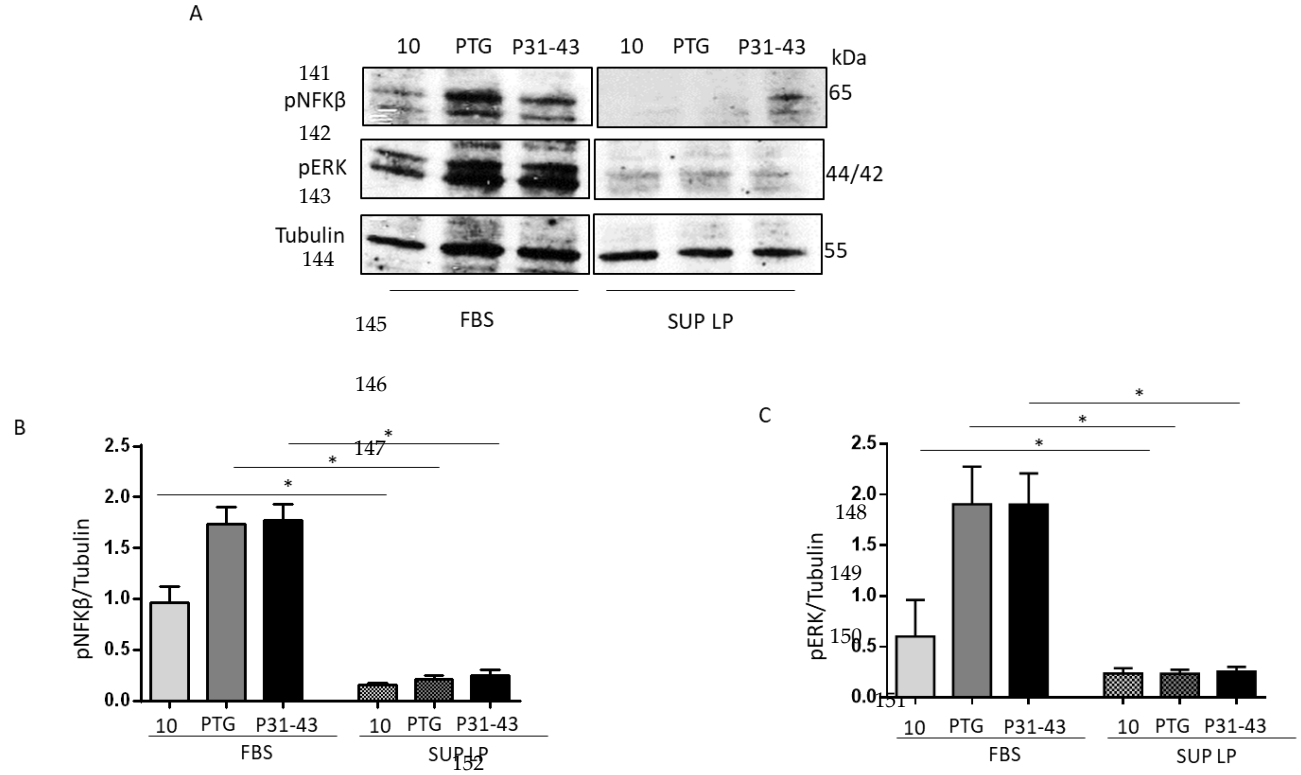
Authophagy markers LC3II, and p62 protein, a protein necessary for autophagy progression were evaluated before and after treatment with gliadin peptides, PTG and P31-43, by western blotting in CaCo-2 cells. We observed a reduction of LC3II levels and an increase of p62 indicating that both the PTG and P31-43 can reduce the authophagy and its progression. Interestingly pretreatment with post- biotic of LP for 3 h prevent effects of gliadin peptides on LC3 and p62 levels. (Figure 2A-C) Moreover, these data were confirmed also by immunofluorescence. (Figure 2 D)



Pretreatment with postbiotc from LP decreased NFK-β and ERK phosphorylation induced by treatment with P31-43 and PTG

Both PTG and P31-43 are able to activate NFKβ [15 and la review]and PTG has been described to activate mTOR (Lavoro di Monteleone).We have tested the effect of the LP postbiotc on PTG and P31-43 inflammation markers increase. For this purpose, CaCo-2 cells were pretreated with postbiotcs of LP for 1 h and after stimulated with PTG and P31-43, activation of NFKβ and ERK was evaluated by WB. Our data showed an increase NFKβ phosphorylation levels after stimulation with PTG and P31-43. Postbiotcs of LP was able to reduce this phosphorylation(Figure 3 A and B). Since MAPKs are involved in Iκκ-dependent NF-κB activation [27]. Also ERK activation was increased

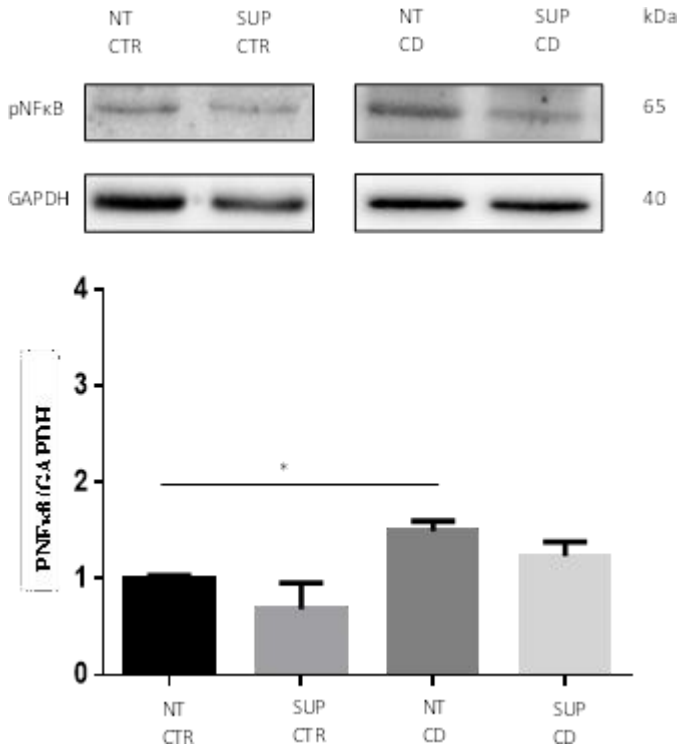
after PTG and P31-43 treatment as shown before [15] and, again, preatreatment with postbiotcs of LB prevented ERK activation (Figure 3 A and C).

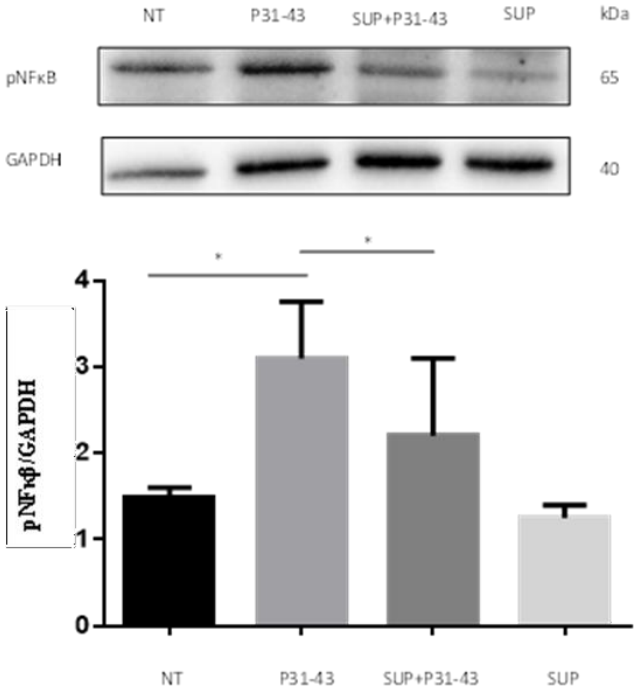


In CD intestinal organoids Lactobacillus paracasei decreased NFK-β phosphorylation and prevented P31-43 effects.

In other to test postbiotc LP in a more complex and physiologic cell model we have used intestinal organoids derived from CD patient in the active phase of the disease and control subjects. Organoids were derived from intestinal staminal cells and were cultivated for 4 weeks in 3D before opening them in 2D to allow treatment on the apical part of the epithelial cells.

Intestinal organoids from CD patients are inflamed (REF IJMS 2021 submitted) respect to controls as shown in figure 4 A and B. In these condition we tested the effect of the LP post- biotic. Interestingly the LP postbiotc was able to reduce the constitutive inflammation present in CD organoids.





Next we tested the effect of P31-43 on CD organoids using as read out of inflammation the phosphorylation of NFkB. As shown in figure 4 C and D P31-43 was able to increase

NFkB phosphorylation in a statistical significant, this effect was already described before (REF). LP postbiotc was able to prevent the increase of NFkB phosphorylation induced by P31-43 on CD organoids.

Material and methods

Cell cultures and treatments

Human colon adenocarcinoma-derived cells (CaCo-2) obtained from Interlab Cell Line Collection (Centro di Biotecnologie Avanzate, Genoa, Italy) were grown in DMEM supplemented with 10% foetal calf serum, 100 units penicillin-streptomycin/mL, and 1 mmol/L glutamine (All these products are Gibco Invitrogen, Milan, Italy). Cells were maintained in a humidified atmosphere (95%) of air and 5% CO2 at 37°C. LPS-free synthetic peptide P31-43 (Inbios, >95% purity, MALDI-toff analysis as expected) was obtained by Ultrasart-D20 (Sartorius AG, Goettingen, Germany) filtration. LPS levels were below detection (<0.20 EU/mg), assessed by commercial QCL-1000 kit (Cambrex Corporation, New Jersey USA). P31–43 sequence: LGQQQPFPPQQPY. Peptic- tryptic -digest of A gliadin were obtained as described before . The alcohol-soluble protein fraction was extracted from whole flour from wheat (Triticum aestivum, variety Sagittario) and then submitted to digestion as previously described . Prolamin peptic–tryptic (PT) digests indicated as PTG were freeze- dried, lyophilized, and stored at 20C until used.

Gliadin peptides were used in the following concentrations: Peptic-tryptic digests of gliadin (PTG) 500 μg/ml and P31-43 peptide was used at 100 μg/ml.

Organoids

One to two duodenal biopsies per individual were taken with standard endoscopic EGDS during routine gastroduodenoscopy and placed in ice-cold 10 ml isolation buffer (5.6 mmol/L Na2HPO4 (Sigma S7907; Sigma–Aldrich, St Louis, MO), 8.0 mmol/L KH2PO4 (Sigma P5655; Sigma–Aldrich), 96.2 mmol/L NaCl (Sigma S5886; Sigma–Aldrich), 1.6 mmol/L KCl (Sigma P5405; Sigma–Aldrich), 43.4 mmol/L sucrose (Fisher BP220-1; Thermo Fisher Scientific, Waltham, MA), and 54.9 mmol/L D-sorbitol (Fisher BP439-500; Thermo Fisher Scientific)) in deionized water. Crypt units were isolated according to the protocol of Yuli Wang et al. 32 with minor variations. Briefly, after 60 minutes, the biopsy samples were further enzymatically digested with collagenase (2 mg/ml, Sigma–Aldrich Milan, Italy) in washing buffer (WB) containing penicillin (100 units ml−1), streptomycin (0.1 mg ml−1), l-glutamine (2 mM) and FBS (10%, vol/vol) in DMEM/F12 with HEPES on ice for 30 minutes. The digest was filtered through a 70 μm strainer (Falcon, Germany) and the strainer was rinsed with an additional 10 ml of WB. Crypts were collected by centrifugation at 500×g for 5 min. The supernatant was discarded, the crypts were carefully resuspended in 40 μl of ice-cold Matrigel matrix (Corning 35623 Milan, Italy) to enable three-dimensional growth in 48-well plates; the plates were incubated in a cell culture incubator at 37 °C and 5% carbon dioxide for 10 minutes to allow the Matrigel to solidify. Afterwards, 300 μl cell culture medium enriched with supplements (CM-S) was added to each well and was replaced every second day. The organoids were used for assays or cryo-preserved at −150 °C. To cryopreserve organoids, they were washed with ice-cold PBS EDTA to re-move Matrigel and collected by

centrifugation at 500×g for 5 min. Organoid pellets were suspended in 1 ml WB, 10% faecal calf serum (FCS, Biochrom, Germany), and 10% dimethyl sulfoxide, slowly frozen to −80 °C in a cryo freezing container (Nalgene, Germany), and then transferred to −150 °C for long-term storage. For further research, the cryopreserved organoids were quickly thawed at 37 °C, transferred to 10 ml WB, centrifuged at 2000×g for 5 min, plated with Matrigel and cultured in CM-S medium. For 2D organoids, organoids were seeded in six wells pretreated with Matrigel diluted 1:40 in phosphate-buffered saline (PBS)

Culture medium to maintain organoids (CM-S).

Mouse L-cells that expressed Wnt3a, R-spondin, and noggin were commercially purchased (ATCC CRL-3276, Bio Tech Standards, Germany), and a conditioned medium (L-WRN) was prepared ac-cording to the instructions and protocol of the manufacturer. Culture medium with supplements (CM-S) was prepared using 50% conditioned L-WRN medium and 50% fresh primary culture media: Advanced DMEM/F-12 [Invitrogen] 1 mM N-acetyl-L-cysteine (Sigma, Germany), 1x N-2 supple-ments (Gibco, Germany), 1 × B-27® supplements (Gibco, Germany), 50 ng/ml epidermal growth fac-tor, 10 mM nicotinamide (Sigma, Germany), 10 nM Leu15-gastrin I (Sigma, Germany), 500 nM A8301 (inhibitor for ALK4/5/7; Sigma, Germany), 10 μM SB202190 (p38 MAP kinase inhibitor; Sigma, Germany), and 10 μM Y-27632 (p160 ROCK inhibitor; Tocris, Germany) in accordance with the pro-tocols of Sato et al. 33, VanDussen et al. 34 and Yuli Wang et al. 32. The organoids were cultured with 300 μl culture medium, which was changed every second or third day.

Bacterial growth conditions

To prepare the postbiotic, we cultivated L. paracasei-CBA L74 in DMEM supplemented with 10% fetal calf serum (FCS, GIBCO), and 1-mM glutamine (GIBCO) until 109 CFU/ml as previously described (Sarno et al., 2014). The bacterial culture was then centrifuged at 3,000 rpm for 10 min and the supernatant was filtered through a 0- to 22-micron filter.

LC3 immunofluorescence staining

Cells, grown on sterile glass coverslips, were transferred into a 24-well plate and pretreated and not with the postbiotcs for 2 h and then stimulated with PTG and P3-43 for 1h. After fixation with 3% paraformaldehyde for 5 min at room temperature and permeabilisation with 0.2% Triton (Biorad, Milan, Italy) for 5min at room temperature, CaCo-2 cells were stained for 1 h at room temperature with anti-LC3II (Cell signaling Milan Italy) antibody. Secondary antibodies Alexa-488 conjugated (Invitrogen) anti-rabbit for LC3II were added to the coverslips for 45 min at room temperature. The coverslips, after mounting on glass slides, were observed by confocal microscope (LSM 510 Zeiss, Milan, Italy), and images were analysed with AIS Zeiss software to evaluate the intensity of fluorescence (FI) of the microscopic field under consideration (Lania et al 2018). Magnification of the micrographs was the same for all the figures shown (63 × objective).

Western Blot

After treatments the CaCo-2 cells were washed twice with cold PBS and resuspended in lysis buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 5 mM MgCl2, 150 mMNaCl, 1% Triton, 1 mM PMSF, 1 mM VO4, 100× Aprotinin, and 50× LAP, all of which were purchased from Sigma, Milan, Italy, except for LAP, which was obtained from Roche, Milan, Italy). The human spheroids were seeded in six multiwells (Corning, Milan, Italy) coated with Matrigel di-luted 1:40 in phosphate buffered saline (PBS) for 3 days. After they were stimulated with P31–43 or PTG and postbiotic were homogenized in tissue homogenization buffer (50 mM Tris–HCl [pH 8], 150 mM NaCl, 5 mM MgCl2, 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM VO4, aprotinin, and LAP. The cell lysates were analysed using SDS-PAGE with a standard running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) and transferred onto nitrocellulose membranes (WhatmanGmbh, Dassel, Germany) using transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% methanol, all of which were purchased from Sigma-Aldrich, Milan, Italy). The membranes were blocked with 5% nonfat dry milk and probed with rabbit anti pmTOR, pp70S6k, p4EBP, LC3II (Cell Signaling, Euroclone, Milan, Italy), rabbit anti-pNF-κB (Elabscience Microtech NaplesItaly), mouse anti pERK(Santa Cruz ,Milan Italy) and mouse antitubulin (Sigma-Aldrich, Milan, Italy). The bands were visualized using ECL (GE Healthcare, Amersham, Buckinghamshire, UK) with exposure times of 2–10 min. The band intensity was evaluated by integrating all the pixels of a band after subtraction of the background to calculate the average of the pixels surrounding the band [35,36].

Statistical analysis

Statistical analysis and graphics were obtained from GraphPad Prism (San Diego, CA). Mean and standard deviation of the experiment was calculated; their significance was evaluated by Student's t-test accepting as significant only results that showed values of $p < 0.05$.

3. Discussion

In this study, we investigated the effects of gliadin on the mTOR/autophagy pathway and inflammation in epithelial cells and intestinal organoids from CD patients. Moreover we have evaluated the ability of the postbiotc Lactobacillus Paracasei (LP) to prevent these effects.

mTOR is a tyrosine kinase that is able to regulate cell proliferation and inhibit the autophagy pathway. It is regulated by signals, such as growth factors and nutrients, that drive cell growth and proliferation [50]. The autophagy pathway is triggered when mTOR is dephosphorylated / inactivated. It is a pathway closely connected with cell regeneration and consists of anauto-cell digestion process generally activated tovremove damaged macromolecules and organelles in order to maintain cellular homeostasis [51]. The activation of mTOR and the consequent deactivation of the autophagy pathway causes the induction of inflammatory markers such as NFkB (REF). (**Life, death, and autophagy in cancer: NF-κB turns upeverywhereInactivation of MTOR promotes autophagy-mediated epithelial injury in particulate matter-induced airway inflammation)**

CaCo-2 cells, intestinal epithelial cells derived from a colon carcinoma and responsive to gliadin, were used to study the effects of gliadin on the mTOR and autophagy

pathway. Both the P31-43 peptide and peptic-tryptic digest of gliadin (PTG) are able to activate mTOR and the downstream pathway by activation of the E4BP and 70S6k proteins. Furthermore, both P31-43 and PTG are able to activate NFkB in CaCo-2 cells. Since the activation of mTOR induces a reduction of autophagy (**REF**), we investigated the activity of P31-43 and PTG on autophagy itself.We have demonstrated that in presence of PTG and P31-43 there is a reduction of LC3-positive vesicle and the autophagic flux to the lysosomes was decreased as demonstrated by the increase in p62. Taken together, these data demonstrate that P31-43 and PTG induce a reduction in both autophagic vesicles and their flux to lysosomes.

Recent studies have investigated the ability of probiotics to prevent the effects of gliadin in vivo. Their effects have been studied both in subjects with celiac disease (CD) and in subjects with potential celiac disease. (Citare review probiotici). Pro-biotics seem to have a good effect on CD sim-yomps when they are present, but they do not seem to have effects on the intestinal lesion.

Taken together, the data on probiotic effects on CD indicate that the use of probiotics can act on gastrointestinal symptoms giving an ameliorative effect but have little effect on the natural history of the disease.

More recently, the interest of the scientific world has focused on postbiotcs. A postbiotic is defined as a "preparation of inanimate microorganisms and/or their components: soluble factors secreted by live bacteria or released after their lysis, including various cell surface components, lactic acid, short-chain fatty acids (SCFA) and bioactive peptides that confers a health benefit on the host". (REF) (**The International Scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics).**

At the present no reports are available about the use of post-bionics in CD. For this reason in this study, we investigated the ability of LP postbiotc to prevent the effects of gliadin and P31-43 peptide on mTOR/autophagy pathways and inflammation in CaCo 2 cells. Moreover we have used intestinal organoids from CD patients to test LP postbiotc effect on inflammation in a more complex and physiologic cell model.

We have shown that, after pretreatment with the postbiotc of LP both the peptide P31-43 and the PTG effects on mTOR activity and the downstream pathway were prevented. Moreover also pNFkB was also reduced in presence of LP postbiotc and PTG or P31-43 .

We investigated the activity of P31-43 and PTG on CaCo-2 cells pretreated with postbiotc LP on autophagy, demonstrating an increase in autophagy vesicles with the increase in the LC 3 marker and of the autophagic flux to the lysosomes with the reduction of p62.

Recently, Freire R et al using intestinal organoids developed from duodenal biopsies from both non-celiac (NC) and celiac (CD) patients have analyzed the role of microbiota-derived molecules in modulating the epithelium's response to gluten. So they selected three bacterial bioproducts: butyrate, lactate, and PSA derived from Bacteroides fragilis All bioproducts exerted a global protective effect by reducing the pro-inflammatory cytokine secretion triggered by PTG.

Using intestinal organoids derived from celiac (CD) patients we have analyzed the effect of P31-43 after pretreatment with LP postbiotc on inflammatory marker NFkB demonstrating a reduction. The use of in vitro patient-derived organoids to model CD pathogenesis could be a novel tool to further study CD treatment and prevention.

In conclusion, the LP postbiotc is able to prevent in vitro the effects of gliadin peptides on the pathway of mTOR, autophagy and NFkB.

These pre-clinical studies are a good basis for activating clinical trials in celiac patients to prevent the pro-inflammatory effects of gliadin peptides. In particular, it would be interesting to test the effect of the postbiotc for its ability to prevent disease in potential subjects, who have anti-tranglutaminase antibodies, but have not yet developed the intestinal lesion typical of celiac disease.

Currently, the only available treatment for a patient with celiac disease is a strict gluten-

free diet, Despite patients' best efforts,some subjects can result in continuous exposure due to cross-contamination or traces of gluten in food. These risks could, in some cases, compromise the health and quality of life of these subjects. It is therefore generally useful to study compounds that can prevent the inflammatory effects of gliadin with the hope of reducing the burden of living with celiac disease and improving long-term health outcomes.

The Materials and Methods should be described with sufficient details to allow others to replicate and build on the published results. Please note that the publication of your manuscript implicates that you must make all materials, data, computer code, and protocols associated with the publication available to readers. Please disclose at the submission stage any restrictions on the availability of materials or information. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited.

Research manuscripts reporting large datasets that are deposited in a publicly available database should specify where the data have been deposited and provide the relevant accession numbers. If the accession numbers have not yet been obtained at the time of submission, please state that they will be provided during review. They must be provided prior to publication.

Interventionary studies involving animals or humans, and other studies that require ethical approval, must list the authority that provided approval and the corresponding ethical approval code.

Figure legends

Figure 1. LP postbiotc prevent gliadin induced activation of mTOR pathway in CaCo-2 cells.

A. Western blot analysis of protein lysates from CaCo-2 cells untreated (NT), treated with PTG and P31-43 for 30 minutes and pretreated with LP postbiotc for 1h were blotted with antibodies against pmTOR, pp70S6k and p4EBP. Tubulin was used as a loading control. The autoradiographs are representative of three independent experiments. B. Densitometric analysis of bands from WB as in A. Columns represent the mean, bars the standard deviation of the relative intensity of pmTOR, pp70S6k and p4EBP respect to total tubulin protein. Student's *t*-test compared to the untreated (NT) sample, $*p < 0.05$.

Figure 2. Pretreatment with LP postbiotc increased LC3II espression after treatment with PTG and P31-43

Western blot analysis of protein lysates from CaCo-2 cells untreated (NT), treated with PTG and P31-43 for 1h and pretreated with LP postbiotc for 2h were blotted with antibodies against LC3II and p62. Tubulin was used as a loading control. The autoradiographs are representative of three independent experiments. B. Densitometric analysis of bands from WB as in A. Columns represent the mean, bars the standard deviation of the relative intensity of LC3II and p62 respect to total tubulin protein. Student's *t*-test compared to the untreated (NT) sample, $*p < 0.05$.

C. Immunofluorescence analysis of anti-LC3II from untreated CaCo-2 cells and CaCo-2 cells treated with PTG and P31-43 for 1h minutes and pretreated with LP for 2h. Images obtained using a 63 x objective (2 times digital zoom) are shown. White bar represent 5 micrometers

Figure 3. Pretreatment with LP decreased NFK-β phosphorylation after treatment with P31-43 and PTG

A. Western blot analysis of protein lysates from CaCo-2 cells untreated (NT), treated with PTG and P31-43 for 30 minutes and pretreated with LP postbiotc for 1h were blotted with antibodies against pNFKβ and pERK. Tubulin was used as a loading control. The autoradiographs are representative

of three independent experiments. B. Densitometric analysis of bands from WB as in A. Columns represent the mean, bars the standard deviation of the relative intensity of pNFKβ and pERK respect to total tubulin protein. Student's *t*-test compared to the untreated (NT) sample, $*p < 0.05$.

Figure 4

Inflammation in CD organoids can be prevented by LP post biotic

A. **Western blot analysis of p**rotein lysates from organoids from CD patients and controls, treated with LP postbiotc for 3h were blotted with antibodies against NfkB. Tubulin was used as a loading control. The autoradiographs are representative of three independent experiments. Number of subjects investigated was 3. B. Densitometric analysis of bands from WB. Columns represent the mean, bars the standard deviation of the relative intensity of NFkB respect to total tubulin protein. Student's *t*-test compared to the untreated (NT) sample, $*p < 0.05$.

C. **Western blot analysis of p**rotein lysates from organoids from CD patients and controls, treated with P31-43 for 1h and pretreated with LP postbiotc for 2h were blotted with antibodies against NfkB. Tubulin was used as a loading control. The autoradiographs are representative of three independent experiments. Number of subjects investigated is indicated. B. Densitometric analysis of bands from WB. Columns represent the mean, bars the standard deviation of the relative intensity of NFkB respect to total tubulin protein. Student's *t*-test compared to the untreated (NT) sample, $*p < 0.05$.

Western blot analysis of protein lysates from CaCo-2 cells untreated (NT), treated with P31-43 for 30 minutes and pretreated with LP postbiotc for 1h were blotted with antibodies against NfkB. Tubulin was used as a loading control. The autoradiographs are representative of three independent experiments. B. Densitometric analysis of bands from WB as in A. Columns represent the mean, bars the standard deviation of the relative intensity of pmTOR, pp70S6k and p4EBP respect to total tubulin protein. Student's *t*-test compared to the untreated (NT) sample, $*p < 0.05$.**Data Availability Statement:** In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section "MDPI Research Data Policies" at https://www.mdpi.com/ethics. If the study did not report any data, you might add "Not applicable" here.

References

1. [1] S.K. Arora, P. Dewan, P. Gupta, Microbiome: Paediatricians' perspective, Indian J Med Res 142(5) (2015) 515-24.
2. [2] A.T. Vieira, M.M. Teixeira, F.S. Martins, The role of probiotics and prebiotics in inducing gut immunity, Front Immunol 4 (2013) 445.
3. [3] P. Louis, C.P. O'Byrne, Life in the gut: microbial responses to stress in the gastrointestinal tract, Sci Prog 93(Pt 1) (2010) 7-36.
4. [4] N. Kamada, G.Y. Chen, N. Inohara, G. Núñez, Control of pathogens and pathobionts by the gut microbiota, Nat Immunol 14(7) (2013) 685-90.
5. [5] D. Artis, Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut, Nat Rev Immunol 8(6) (2008) 411-20.
6. [6] J.M. Wells, O. Rossi, M. Meijerink, P. van Baarlen, Epithelial crosstalk at the microbiota-mucosal interface, Proc Natl Acad Sci U S A 108 Suppl 1(Suppl 1) (2011) 4607-14.
7. [7] C.U. Duerr, M.W. Hornef, The mammalian intestinal epithelium as integral player in the establishment and maintenance of host-microbial homeostasis, Semin Immunol 24(1) (2012) 25-35.
8. [8] V. D'Argenio, G. Casaburi, V. Precone, C. Pagliuca, R. Colicchio, D. Sarnataro, V. Discepolo, S.M. Kim, I. Russo, G. Del Vecchio Blanco, D.S. Horner, M. Chiara, G. Pesole, P. Salvatore, G. Monteleone, C. Ciacci, G.J. Caporaso, B. Jabrì, F. Salvatore, L. Sacchetti, Metagenomics Reveals Dysbiosis and a Potentially Pathogenic N. flavescens Strain in Duodenum of Adult Celiac Patients, Am J Gastroenterol 111(6) (2016) 879-90.

9. [9] G. Labruna, M. Nanayakkara, C. Pagliuca, M. Nunziato, L. Iaffaldano, V. D'Argenio, R. Colicchio, A.L. Budelli, R. Nigro, P. Salvatore, M.V. Barone, L. Sacchetti, Celiac disease-associated Neisseria flavescens decreases mitochondrial respiration in CaCo-2 epithelial cells: Impact of Lactobacillus paracasei CBA L74 on bacterial-induced cellular imbalance, Cell Microbiol 21(8) (2019) e13035.
10. [10] M.V. Barone, A. Gimigliano, G. Castoria, G. Paolella, F. Maurano, F. Paparo, M. Maglio, A. Mineo, E. Miele, M. Nanayakkara, R. Troncone, S. Auricchio, Growth factor-like activity of gliadin, an alimentary protein: implications for coeliac disease, Gut 56(4) (2007) 480-8.
11. [11] M.V. Barone, R. Troncone, S. Auricchio, Gliadin peptides as triggers of the proliferative and stress/innate immune response of the celiac small intestinal mucosa, Int J Mol Sci 15(11) (2014) 20518-37.
12. [12] M. Morita, S.P. Gravel, L. Hulea, O. Larsson, M. Pollak, J. St-Pierre, I. Topisirovic, mTOR coordinates protein synthesis, mitochondrial activity and proliferation, Cell Cycle 14(4) (2015) 473-80.
13. [13] S. Sedda, V. Dinallo, I. Marafini, E. Franzè, O.A. Paoluzi, R. Izzo, P. Giuffrida, A. Di Sabatino, G.R. Corazza, G. Monteleone, mTOR sustains inflammatory response in celiac disease, Sci Rep 10(1) (2020) 10798.
14. [14] M. Nanayakkara, G. Lania, M. Maglio, V. Discepolo, M. Sarno, A. Gaito, R. Troncone, S. Auricchio, R. Auricchio, M.V. Barone, An undigested gliadin peptide activates innate immunity and proliferative signaling in enterocytes: the role in celiac disease, Am J Clin Nutr 98(4) (2013) 1123-35.
15. [15] M. Nanayakkara, G. Lania, M. Maglio, R. Auricchio, C. De Musis, V. Discepolo, E. Miele, B. Jabri, R. Troncone, S. Auricchio, M.V. Barone, P31-43, an undigested gliadin peptide, mimics and enhances the innate immune response to viruses and interferes with endocytic trafficking: a role in celiac disease, Sci Rep 8(1) (2018) 10821.
16. [16] M.C. Maiuri, D. De Stefano, G. Mele, S. Fecarotta, L. Greco, R. Troncone, R. Carnuccio, Nuclear factor kappa B is activated in small intestinal mucosa of celiac patients, J Mol Med (Berl) 81(6) (2003) 373-9.
17. [17] I. Nakagawa, A. Amano, N. Mizushima, A. Yamamoto, H. Yamaguchi, T. Kamimoto, A. Nara, J. Funao, M. Nakata, K. Tsuda, S. Hamada, T. Yoshimori, Autophagy defends cells against invading group A Streptococcus, Science 306(5698) (2004) 1037-40.
18. [18] B. Levine, N. Mizushima, H.W. Virgin, Autophagy in immunity and inflammation, Nature 469(7330) (2011) 323-35.
19. [19] K. Kirkegaard, M.P. Taylor, W.T. Jackson, Cellular autophagy: surrender, avoidance and subversion by microorganisms, Nat Rev Microbiol 2(4) (2004) 301-14.
20. [20] A. Chargui, M.V. El May, Autophagy mediates neutrophil responses to bacterial infection, Apmis 122(11) (2014) 1047-58.
21. [21] C.F. Bento, N. Empadinhas, V. Mendes, Autophagy in the fight against tuberculosis, DNA Cell Biol 34(4) (2015) 228-42.
22. [22] T. Wang, K. Liu, L. Wen, Y. Yang, X. Yin, K. Liu, Y. Chen, Y. He, M. Yang, Y. Wei, B. Wang, D. Chen, Autophagy and Gastrointestinal Diseases, Adv Exp Med Biol 1207 (2020) 529-556.
23. [23] R.S. Rekha, S.S. Rao Muvva, M. Wan, R. Raqib, P. Bergman, S. Brighenti, G.H. Gudmundsson, B. Agerberth, Phenylbutyrate induces LL-37-dependent autophagy and intracellular killing of Mycobacterium tuberculosis in human macrophages, Autophagy 11(9) (2015) 1688-99.
24. [24] N. Mizushima, M. Komatsu, Autophagy: renovation of cells and tissues, Cell 147(4) (2011) 728-41.
25. [25] S.R. Yoshii, N. Mizushima, Monitoring and Measuring Autophagy, Int J Mol Sci 18(9) (2017).
26. [26] M. Conte, M. Porpora, F. Nigro, R. Nigro, A.L. Budelli, M.V. Barone, M. Nanayakkara, Pro-pre and postbiotic in celiac disease, Applied Sciences (Switzerland) 11(17) (2021).
27. [27] J.H. Shi, S.C. Sun, Tumor Necrosis Factor Receptor-Associated Factor Regulation of Nuclear Factor κB and Mitogen-Activated Protein Kinase Pathways, Front Immunol 9 (2018) 1849.