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Project partners/consortium:
P1 – University of Split – UNIST (coordinator)
P2 – Green Environmental Research Ltd. – GREENER
P3 – Centaurus d.o.o. – CENTAURUS
P4 – Alma Mater Studiorum Università di Bologna – UNIBO (vice-coordinator)
P5 – Universita' Cattolica del Sacro Cuore – UCSC
P6 – Martino Rossi S.p.A. – MROSSI
P7 – Spanish National Research Council – CSIC
P8 – DOMCA SAU – DOMCA
P9 – Cukurova University – CUNI
P10 – Chouaib Doukkali University – UCD
P11 – Sidi Mohamed Ben Abdellah University – USMBA

Deliverable 2.2. Report on the use of *in vitro* digestion to assess the functional properties of the selected innovative ingredients

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1. Aim of the work and summary

This report presents the work carried out and the results obtained by **partner 7 (P7) CSIC-CIAL** during WP2 activities. The main objective in this phase is the selection of the best source of bioactive ingredients. To achieve this, two experimental approaches involving gastrointestinal digestions and colonic fermentations were carried out, according to the tasks proposed in the InnoSol4Med project (*"Task 2.5. Assessment of functional properties of the selected innovative ingredients during in vitro digestion"*). According to the comparison of the gastrointestinal digestion and colonic fermentation of the different extracts studied (7 in total) and the analysis of gut microbiota, microbial metabolism, and intestinal barrier integrity, the results suggest that the Yellow onion extract is the ingredient with the most interesting functional properties and will therefore be proposed for the next phase of the InnoSol4MedFood PRIMA project.

2. Report on the activities of Task 2.5: Assessment of functional properties of the selected innovative ingredients during *in vitro* digestion

2.1 Materials and methods

2.1.1. Material provided by the project partners

Table 1 shows the different products provided by the project partners.

Table 1. Information about samples provided by project partners.

Supplier partner (P)	Products	Units	Reception date
Domca-CTA (P8)	Cumin extract	1x2ml	18/1/24
Domca-CTA (P8)	Onion extract	1x2ml	18/1/24
Domca-CTA (P8)	Leek extract	1x2ml	18/1/24
University Department of Marine Studies, University of Split (P1)	Yellow onion extract	1x5g	3/1/24
DISTAL - Alma Mater Studiorum – Università di Bologna (P4)	Cinnamon essential oil	2x1ml	7/12/23
DISTAL - Alma Mater Studiorum – Università di Bologna (P4)	Clove essential oil	2x1ml	7/12/23
DISTAL - Alma Mater Studiorum – Università di Bologna (P4)	Oregano essential oil	2x1ml	7/12/23

2.1.2. Selection of the test doses for simulation assays

The test doses of different products for simulation assays (**Figure 1**) were selected according to their solubility and bioactivity, as well as the information provided by the project partners and the revised scientific literature.

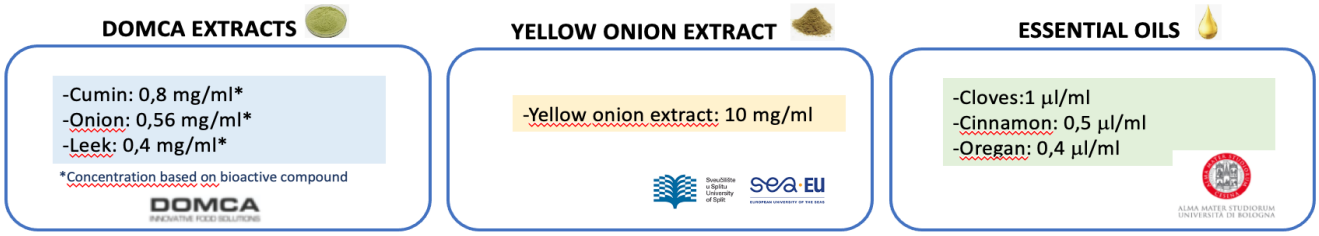

Figure 1. Schematic view of the test dose of different products for simulation assays. DOMCA EXTRACTS (P8); YELLOW ONION EXTRACT (P1); ESSENTIAL OILS (P4)

2.1.3. Gastrointestinal digestions

Gastrointestinal digestions were carried out according to the INFOGEST protocol (Brodkorb et al., 2019), with some optimizations by our group. As a previous step, the enzymatic activities of the enzymes used in gastrointestinal digestion are evaluated. According to the results obtained, **Figure 2** shows the concentrations of enzymes (mg/mL) to be used in the oral, gastric and intestinal stages of digestion.

Digestion stage	Enzymes	Name, Provider (Code)	Concentration in SSF (mg/mL)	Concentration in SGF (mg/mL)	Concentration in SIF (mg/mL)
Oral	α-amylase	Amylase from Human Saliva, SigmaAldrich (A1031)	0.108 mg/mL	-	
Gastric	Pepsine + Gastric lipase	Rabbit gastric extract, Lipolytech (RGE15)	-	7.06 mg/mL	-
Intestinal	Pancreatine	Pancreatine from porcine pancreas x8USP, SigmaAldrich (P7545)	-	-	16.9 mg/mL
Intestinal	Bile	Bovine Bile, SigmaAldrich (B3883)	-	-	11.7 mg/mL

Figure 2. Information about de enzymes used during different stages of gastrointestinal digestion. SSF: saliva solution fluid; SGF: gastric solution fluid; SIF: intestinal solution fluid.

After that, gastrointestinal digestions of the study products were carried out. The most relevant steps of this process are shown in **Figure 3**.

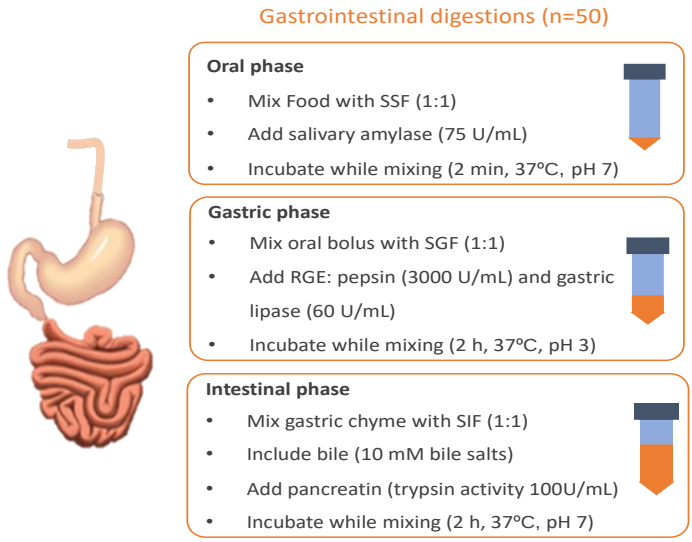

Figure 3. Most relevant steps in gastrointestinal digestion process according to INFOGEST protocol.

At the end of intestinal digestion, the digests were keep at -80 °C until further assessment for colonic fermentations.

2.1.4. Colonic fermentations

Intestinal digests from the gastrointestinal digestion were subjected to static colonic fermentations with human faecal microbiota (**Figure 4**). A total of three independent colonic fermentation experiments (DOMCA extracts, yellow onion extract and essential oils) with their corresponding controls were carried out.

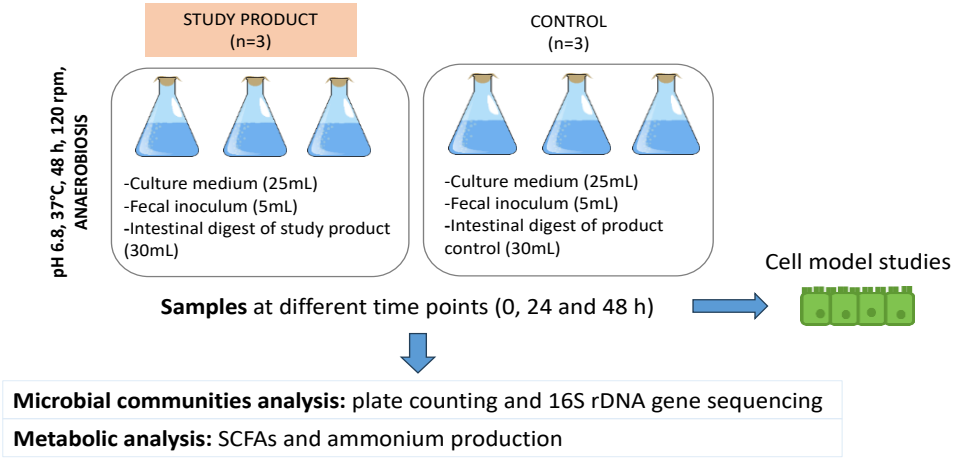

Figure 4. Schematic view of colonic fermentation experimental design and samples collection.

Each flask contained 30 mL of intestinal digest, 50 mL of colon nutrient medium (CNM) (Gil-Sánchez et al., 2017), and 5 mL of a faecal slurry prepared as described in Tamargo et al. (2022). All fermentation flasks were incubated for 48 h and 120 rpm, simulating the conditions of the distal region of the human large intestine (pH 6.8, 37 °C and anaerobic atmosphere) (Gil-Sánchez et al., 2017). Fermentations were carried out in triplicate and samples were collected at 0, 24 and 48 h. An immediately collected sample aliquot (1 mL) was used for microbial counts. Other aliquots were centrifuged at 10,000 rpm at 4 °C for 10 min. Supernatants were separated and filtered through 0.22 µm PVDF filters (Symta, Spain) and kept at -80 °C until further analysis of microbial-derived metabolites and cell culture experiments. Pellets were kept at -80 °C until DNA extraction.

2.1.5 Microbial community analyses for in vitro colonic fermentations

2.1.5.1 Microbial plate counting

Immediately after sampling, tenfold serial dilutions of the content of each colonic fermentation flask were plated on different types of selective media as described by Tamargo et al. (2018). Plate counting was done in triplicate and data were expressed as log of colony-forming units per millilitre (CFU/mL). Analyses were carried out in triplicate.

2.1.5.2. DNA extraction, 16S ribosomal DNA sequencing and data processing

Pellets of 2 mL sample from each colonic fermentation flask were used for DNA extraction, using the QIAmp® Fast DNA Stool MiniKit (Qiagen, Germany), following the manufacturer's recommendations, with some modifications (Molinero et al., 2022). Briefly, bacterial pellets (both from faecal samples and simgi® colonic compartments) were resuspended in 200 µL of lysis solution (prepared with Tris-HCl pH 8 20 mM, EDTA 2 mM, and Triton x 100 1.20%). Prior to incubation, lysozyme 20 mg/mL, 4 µL of mutanolysin (5KU/mL), and 2.5 µL of lysostaphin (1 mg/mL) (all purchased from Sigma-Aldrich, Merck Life Science) were added to the solution. The mixture was incubated for 1 h at 37 °C. After that, 500 µL of InhibitEX buffer from the Qiagen kit was added, and the contents were transferred into screw-in Eppendorf tubes with zirconia beads (each bead measuring 1 mm and weighing 0.4 g with a ~0.4 mm diameter) to treat the contents in a Fast Prep (FastPrep-24TM, MP Biomedicals) at a speed of 5.5 for 30 sec three times. The tubes were centrifuged for 1 min at maximum speed and 400 µL of supernatant was taken. Then, 25 µL of proteinase K from the kit was added followed by 400 µL of AL buffer. The mixture was incubated for 15 min at 70 °C. Subsequently, 400 µL of

molecular grade ethanol was added. The complete volume was transferred to the Qiagen column and then centrifuged for 1 min at maximum speed. The protocol was completed following the manufacturer's instructions, and the DNA was eluted in 50 µl of Sigma water. The DNA concentration was measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

The DNA concentration obtained in each sample was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, USA). Subsequently,the V3-V4 region of the 16S ribosomal RNA gene was amplified using the following primers: forward 5′-CCTACGGGNBGCASCAG-3′ and reverse 5′-GACTACNVGGGTATCTAATCC-3′. The Illumina® two-step PCR protocol was followed for library preparation, and sequencing was performed on an Illumina® MiSeq instrument (Illumina®, USA) performing 2 × 300 bp paired-end reads. RStudio 2023.06.2 software was used to process the files with raw reads. Taxonomic assignment was performed using the naïve Bayesian classifier implemented in DADA2 using Silva v.138 as a reference database (Quast et al., 2013). Biodiversity, expressed in terms of α-diversity, was estimated using the ASVs by calculating the Observed, Shannon, and Simpson indices through the “Phyloseq” package. The β-diversity was assessed by employing a Bray-Curtis dissimilarity matrix represented by non-metric multidimensional scaling (NMDS). Relative abundances of each taxon were calculated for each sample at the phylum, family, and genus level.

2.1.6. Microbial functionality analysis

2.1.6.1. Analysis of short chain fatty acids (SCFA)

Microbial production of SCFAs in colonic fermentation samples was analysed by gas chromatography coupled to a flame ionization detector (GC-FID) following the method previously reported by García-Villalba et al. (2012). Briefly, 50 µL of sample supernatant was acidified with 0.5% phosphoric acid (200 µL) and mixed with 100 µL of internal standard (2-methylvaleric acid, Sigma) (1.97 mM). The mixture was extracted with 1 mL of n-butanol and introduced into the apparatus for analysis. Quantitative data were obtained by calculating the peak area of each compound relative to that of the internal standard (2-methylvaleric acid). Analyses were performed in triplicate.

2.1.6.2. Ammonium ion production

To determine the proteolytic activity of the microbiota, the ammonium concentrations in colonic fermentation samples were determined using the photometric Spectroquant® ammonium reagent test (Merck,USA). The results, measured at 690 nm, were obtained from a linear regression plot constructed using ammonium standard solution (Sigma-Aldrich, Merck, USA) whose linear range of interest was between 2 and 75 mg NH_4^+/L. Samples were diluted using Milli-Q water to adjust its concentration to the kit's measurement range. Analyses were performed in duplicate.

2.1.7. Cell culture conditions and intestinal barrier integrity assay

Human colonic adenocarcinoma cells, Caco-2 (ATCC HTB-37^{TM}) were cultured in DMEM supplemented with 20% v/v FBS, 1 mM sodium pyruvate and 1% v/v penicillin/streptomycin. Cells were seeded in CELLTREAT® 0.4 µm Polyethylene Membrane Inserts (STEMCELL Technologies) at a density of 1.5 x 10^5 cells/well and were incubated incubated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v FBS and 1% penicillin/streptomycin for 21 days to allow differenciation, establishing a competent intestinal barrier model. Medium was changed twice a week. At day 21, some cells were treated with a inflammatory cocktail (100 µg/mL LPS, 100 ng/mL IL-1β + 50 ng/mL IFNγ + 100 ng/mL TNF-α) for 24 h [IC] to mimic chronic intestinal barrier dysfunction associated with chronic inflammation; while some others were not altered [NC: negative control]. From 24 to 48h of treatment, some barriers were replaced with serum-free media (all NC & part of IC); while some other IC were treated with filtered-supernatants from gastrointestinal simulation of yellow onion extract at 48h and simulation control at 48 hdiluted 1/10 in serum-free media for 24 h. Seeding on inserts allows measuring transepithelial electrical resistance (TEER) across a celular monolayer, providing a quantitative *in vitro* assessment of cells barrier function. TEER was measured at least once per week to check the state of the intestinal barrier model during differentiation, and every 24h between cell-treatments. The blank value was subtracted from the total resistance of each insert and . the net resistance (Ω*cm^2) was calculated by multiplying the sample resistance by the effective area of the membrane (0.33 cm^2 for 24-well inserts).

$$\boldsymbol{TEER}\ (\boldsymbol{\Omega} * \boldsymbol{cm^2}) = (\text{measured TEER } (\Omega) - \text{blank TEER } (\Omega)) \times \textit{membrane insert area } (cm^2)$$

2.1.8. Statistic analysis

Statistical analyses were performed using the XLSTAT Statistic software for Microsoft Excel, 2023.1.6 statistical package (Addinsoft-SARL., USA). A two-way ANOVA and the Games-

Howell post hoc test were used to assess differences on microbial features at different colonic fermentations times (microbial counts, metataxonomic results, phenolic metabolites content and SCFAs production). A two-way ANOVA test with Tukey post hoc correction was used to evaluate differences on ammonium production and intestinal barrier integrity assay. Significant differences were calculated considering $p < 0.05$ for all the analyses. Further, from a microbiological perspective, plate counting values were considered significantly different when Δ log (CFU/mL) ≥ 1, due to plate counting limitations (Gil-Sánchez et al., 2018).

2.2. Results

2.2.1. Effect of the ingredientes on human colonic microbiota

Plate counting results

The impact of different study products on the growth of microbiota during colonic fermentation was evaluated by plate counting as a first approximation. Furthermore, and from a microbiological point of view, differences in values were considered significant when they were both statistically significant and higher than Δ log (CFU/mL) ≥ 1, due to plate counting limitations (Gil-Sánchez et al., 2018; Tamargo et al., 2022). Based on this, **Figure 5** shows the most relevant changes observed during the colonic fermentation assays.

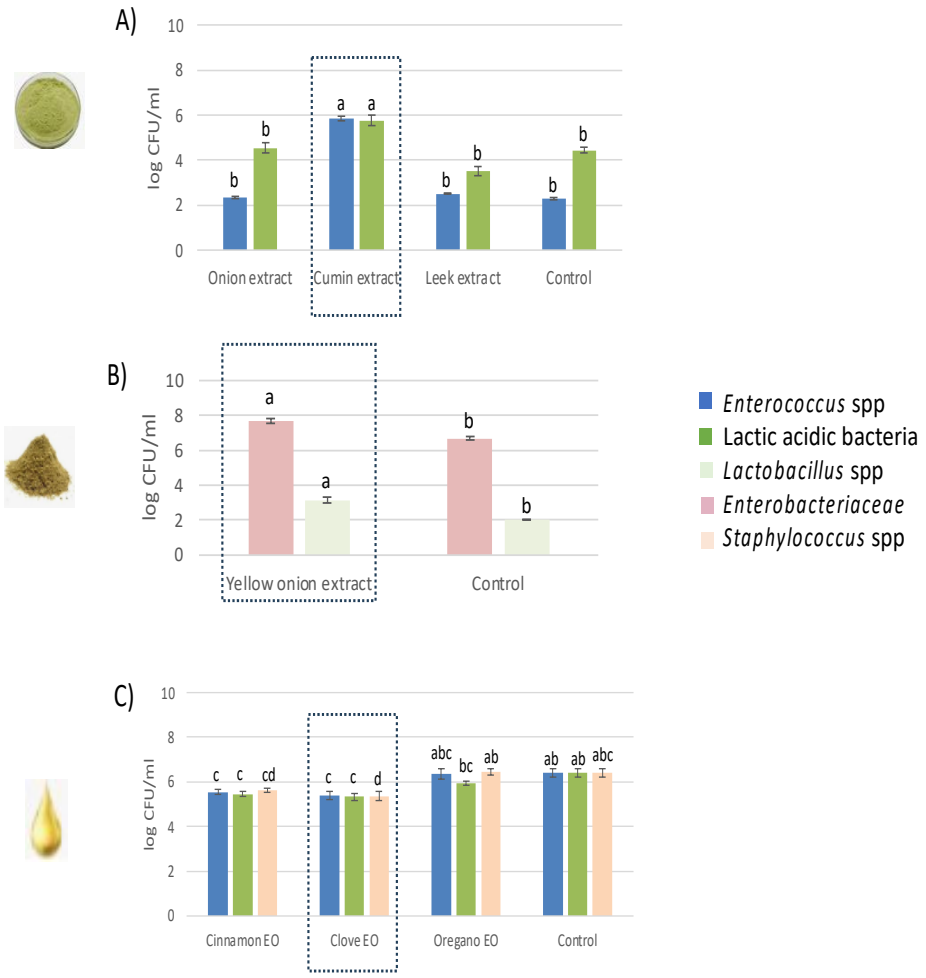

Figure 5. Relevant changes in microbial growth during colonic fermentations using DOMCA extracts, (A, 24 h fermentation), yellow onion extract (B, 48 h fermentation) and essential oils (C, 48 h fermentation), expressed as mean values of log (CFU/mL) ±standard deviation. Different lower-case letters denote statistically significant differences between study products and the control for the same bacterial group. Additionally, the dotted box shows those microbiologically significant change.

Compared to the control, the fermentation of cumin extract showed an increase in the counts of *Enterococcus* spp. and lactic acid bacteria at 24 h of incubation. At 48 h, the yellow onion extract led to an increase in the *Enterobacteriaceae* family and *Lactobacillus* spp. Regard to essential oils, a slight decrease in the growth of *Enterococcus* spp, lactic acid bacteria and *Staphylococcus* spp was observed with clove essential oil. The same trend, although not significant from a microbiological point of view, was observed for the group of *Enterococcus* spp and lactic acid bacteria in the case of cinnamon essential oil. However, in the case of cinnamon essential oil, although significant differences were observed for *Enterococcus* spp. and lactic acid bacteria, they were not significant from a microbiological point of view.

16S rRNA gene sequencing results

Complementarily, changes in microbial populations were also evaluated by 16S rRNA gene sequence analysis. The analysis of the alpha-diversity in terms of observed species, and Shannon and Simpson indexes was carried out. In the case of **DOMCA extracts (Partner 8), i.e. #1 Onion extract; #2 Cumin extract; #3 Leek extract,** not significant differences were found between the extracts and the control for the different times analyzed. However, it was possible to visualize greater biodiversity at 24h in the presence of the three extracts compared to the control, although this effect was not observed at 48h (results not shown). Regarding phylogenetic analysis, results revealed a differential effect of DOMCA extracts with respect to the control depending on the extract.

At the phylum level (**Figure 5**), the results showed an increase in Proteobacteria levels in the presence of Cumin extract with respect to the control at 24h, which was maintained at 48h. Onion and leek extracts also revealed this increase in Proteobacteria levels compared to the control at 48h. On the other hand, in the presence of Cumin and Leek extracts, a decrease in Actinobacteriota levels was observed, and independently, the Cumin extract promoted a decrease in Desulphobacteria and Fusobacteria levels with respect to the control and the rest of the extracts at 48h. It is worth noting the notable increase in the levels of Fusobacteriota at 48h in the presence of the Leek extract. In general, the three DOMCA extracts promoted an increase in Firmicutes levels at 48h compared to the control, as well as Bacteroidota at 24 and 48h (**Figure 5**).

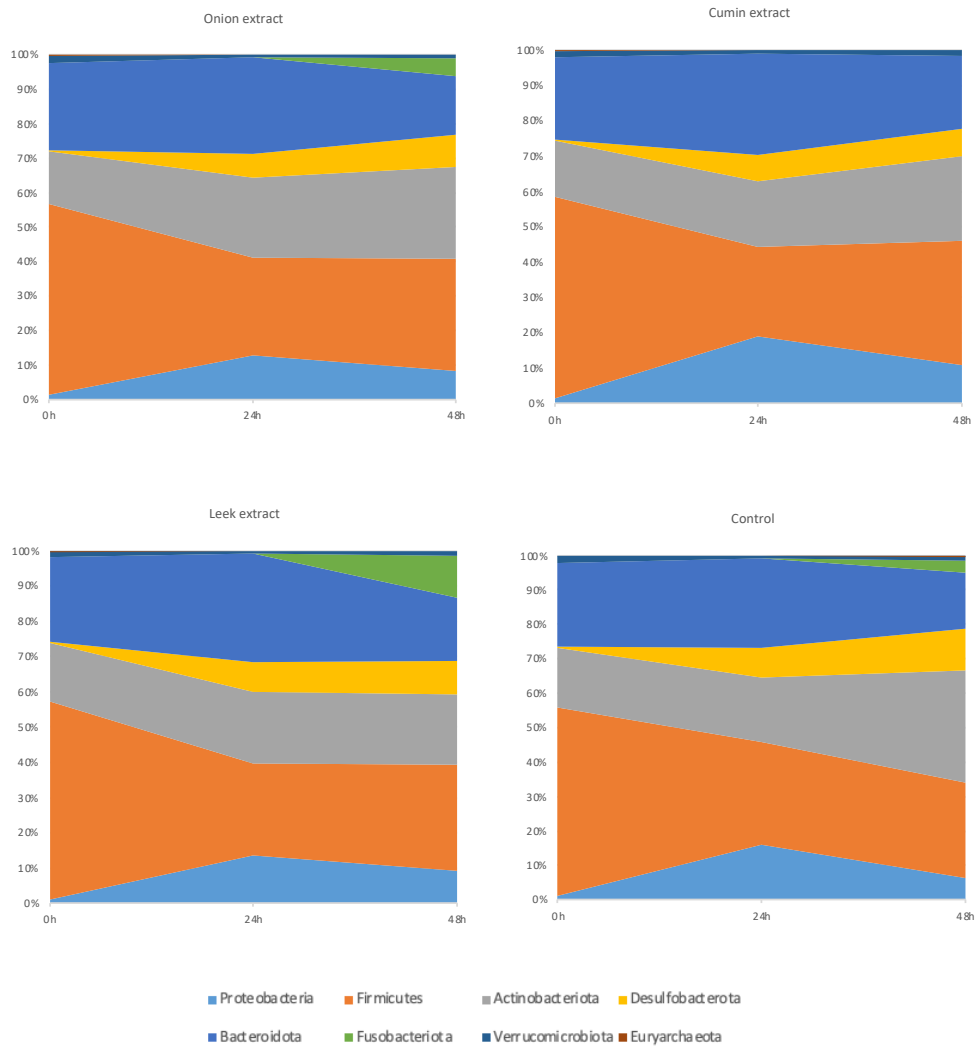

Figure 5. Relative abundance at phylum level for the different DOMCA extracts (Partner 8) and control during colonic fermentations. Graphs show the taxa with a relative abundance > 0.5%.

At the family level, the Onion extract promoted a significant increase in the levels of members of the *Enterococcaceae* and *Lactobacillaceae* families at 24 and 48h with respect to the control, and, although not significant, of members of the *Coriobacteriaceae* family at 24h, although this effect was no longer observed at 48h. On the other hand, Onion extract also seems to promote a decrease in groups associated with positive effects, especially *Lachnospiraceae*. For the rest of the groups, no relevant changes were observed. Regarding the Cumin extract, a significant increase in the levels of members of the *Enterobacteriaceae* and *Enterococcaceae* families was observed at 24 and 48h with respect to the control. The same effect was observed for *Lactobacillaceae* family at 24h of incubation. In addition, at 48 h an increase was also observed

in members of the *Tannerellaceae* family, and although not significant, in other groups associated with negative effects such as *Streptococcaceae* family. Moreover, as with Onion extract, Cumin extract also appeared to promote a decrease in groups associated with positive effects, such as the *Lachnospiraceae* and *Desulfovibrionaceae* families. However, in the presence of Cumin extract, the levels of *Fusobacteriaceae* remained at levels close to 0% relative abundance at 24 and 48h compared to the control, a positive effect considering the pathogenic capacity of this group and its growth advantage in fermentation conditions. On the other hand, at 24 h of fermentation, the Leek extract promoted a significant increase in the levels of members of the *Bifidobacteriaceae* and *Acidaminococcaceae* families as well as a decrease in the levels of *Coriobacteriaceae*. Likewise, at 48 h there was an increase in members belonging to the *Lachnospiraceae* family. The results also showed a decrease in the levels of *Fusobacteriaceae* at 24h with respect to the control, maintaining levels close to 0% relative abundance, however, at 48h a large increase is observed in the presence of the Leek extract with respect to the control, which could be due to some of the components of the extract favouring the growth of this group.

Microbiota analysis at the genus level also showed differences between extracts and the control. In particular, the Onion extract promoted a significant increase in the levels of *Enterococcus* and *Lactobacillus* at 24 and 48h compared to the control. On the other hand, an increase in the presence of Onion extract was observed in the levels of *Collinsella* at 24h, although this effect was not relevant at 48h, showing a clear decrease with respect to the control. The Onion extract also promoted an increase in the levels of *Citrobacter*, *Streptococcus* and *Klebsiella* at 24h, although this was only maintained at 48h in the case of *Citrobacter* and *Streptococcus*. In addition, an increase in *Odoribacter* levels was observed at 48h, among others. Cumin extract promoted a significant increase in the levels of *Escherichia*/*Shigella*, *Enterococcus* and *Citrobacter* at 24 and 48h compared to the control. An increase in the levels of *Lactobacillus* was also observed during the first 24h of fermentation, but this effect was lost at 48h. On the other hand, an increase in *Lachnoclostridium* levels and a decrease in *Collinsella* levels were observed at 48h. Cumin extract also promoted an increase in *Streptococcus* and *Klebsiella* levels at 24h and 48h. Finally, an increase in the levels of *Odoribacter* and *Enterobacter* was also observed at 48h, among others. On the other hand, the Leek extract promoted a significant increase in the levels of members of the genus *Lachnoclostridium* at 48h and *Bifidobacterium* at 24h, accompanied by an increase in the levels of *Phascolarctobacterium* and *Alistipes* at 24 and 48h of fermentation. In this case, the presence of the leek extract caused a decrease in the

levels of *Collinsella* at 24 and 48h, as well as *Fusobacterium* at 24h. Again, the significant increase in the members of this group was observed at 48h. In this case, the increase in *Citrobacter* and *Streptococcus* was also observed at 24 and 48h, and *Klebsiella* at 24h, however, to a lesser extent with respect to the onion and cumin extracts.

In conclusion, it can be highlighted that the modulation of the microbiota exerted by the DOMCA extracts was diverse, affecting both groups associated with positive and negative effects, with no clear trend predominating. However, within this framework, the Leek extract seems to exert the most beneficial effect due to the increase in *Lachnoclostridium*, *Bifidobacterium* and *Alistipes*.

Regard to **Yellow onion extract (Partner 1)**, no significant differences were observed in the alpha-diversity of the microbial communities with respect to the control for the different fermentation times, however, greater biodiversity was found at 24 hours in the presence of yellow onion extract, and lower biodiversity at 48 hours compared to the control (results not shown). Regarding phylogenetic analysis, results revealed that at the phylum level, the feeding with the yellow onion extract promoted an increase in Proteobacteria at 48h of fermentation, along with a decrease in the levels of Desulfobacterota and Fusobacteriota compared to the control. In addition, an increase in members of the Bacteroidota phylum was observed at 24h of fermentation, however, this difference with respect to the control was lost at 48h (**Figure 6**).

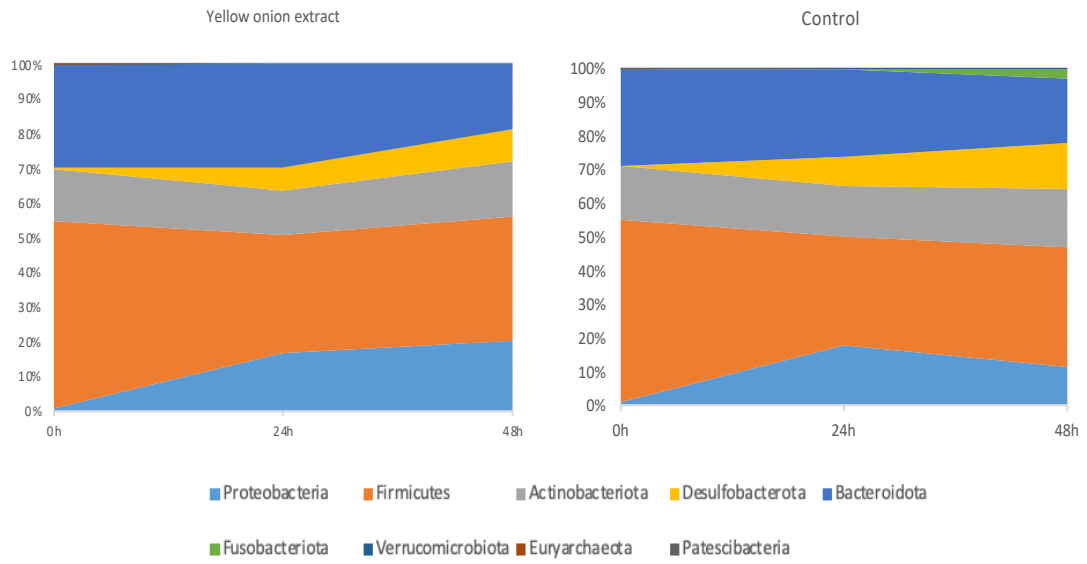

Figure 6. Relative abundance at phylum level for yellow onion extract and control during colonic fermentations. Graphs show the taxa with a relative abundance > 0.5%.

At the family level, the data showed a general decrease in the relative abundance levels of various taxa associated with negative health effects, highlighting members of the families *Enterococcaceae*, *Coriobacteriaceae*, *Fusobacteriaceae*, and *Peptostreptococcaceae*. Likewise, fermentation in the presence of Yellow Onion extract promoted an increase in the levels of *Morganellaceae*, *Erysipelotrichaceae*, and *Erysipelatoclostridiaceae*, as well as members of the family *Ruminococcaceae*. In addition, a decrease in the levels of the family *Lactobacillaceae* was observed, however, despite being significant, the difference in mean relative abundance was not relevant. Finally, at the genus level, the observed differences were characterized by a decrease in the levels of *Enterococcus*, *Lachnoclostridium*, *Collinsella*, *Bilophila*, *Fusobacterium*, *Klebsiella*, and *Terrisporobacter* compared to the control, all of which are taxa associated with negative or pro-inflammatory effects. In addition, yellow onion extract promoted an increase in commensal or beneficial groups, highlighting *Alistipes*, *Agathobacter*, *Subdoligranulum*, *Faecalibacterium*, *Blautia* and *Anaerostipes*.

In general, a positive effect of yellow onion extract on microbial communities was observed, which could be due to its high composition of fiber and flavonoids, which have been related to higher levels of *Faecalibacterium* and *Blautia*, among others.

Regarding **Essential oils (Partner 4),** no significant differences were observed in the alpha-diversity of the microbial communities with respect to the control for the different fermentation times (results not shown). In relation to phylogenetic analysis results indicated that at the phylum level there were no significant changes in any case, however, a tendency was observed for oregano essential oil to decrease the growth of the *Proteobacteria* phylum and increase that of the *Actinobacteria* phylum both at 24 and 48 h of fermentation, which can be considered a positive effect.

At the family level, significant changes were only observed after 24 h of incubation. Specifically, Oregano oil was found to increase the abundance of members of the *Rikenellaceae* family and decrease that of the *Streptococcaceae* family compared to the control. On the other hand, Cinnamon and Clove oils resulted in an increase in members of the *Eggerthellaceae* family. In line with this, results at genus level also showed the ability of Cinnamon and Clove essential oils to promote the growth of *Eggerthella*. Likewise, Cinnamon essential oil and

Oregan essential oil led to an slight increase in the abundance of genus *Desulfovibrio* and *Alistipes*, respectively. Based on these results, it can be concluded that in general, the essential oils do not seem to promote notable modulatory effects on the gut microbiota in the assayed conditions. However, clove essential oil has shown a distinctive effect through the specific enhancement of the genus *Eggerthella*, known for its ability to metabolise polyphenols on substrates rich in these compounds.

2.2.2. Effect of the ingredients on colonic microbiota metabolism

In relation to the functionality of the intestinal microbiota, SCFAs and ion ammonia concentrations are considered indirect indicators of microbial fermentation and metabolism, specifically with the fermentative and proteolytic activities of colonic microbiota, respectively.

2.2.2.1. Fermentative activity of colonic microbiota

As expected, the SFCA concentrations increased during study products colonic fermentation, mostly in the first 24 h but continuing up to 48 h. Compared with the control fermentation, DOMCA extracts (Partner 8) gave rise to a significant increase in the concentration of propionic acid (Leek extract (10,35 mM) > Cumin extract (9,48 mM)> Onion extract (8,57 mM) > control (7,77 mM) at 24 h of incubation (results not showed). The same pattern was observed for this acid at 48 h of incubation, as is showed in **Figure 7 A,** however, only the Cumin and Leek extracts led to a significant change in the propionic production. Likewise, a significant increase in acetic acid with the Leek extract was observed. Regard to butyric acid, no production was observed in the case of Cumin extract. The slightly lower production of SCFA with cumin extract was also reflected in the total fatty acids (results not shown).

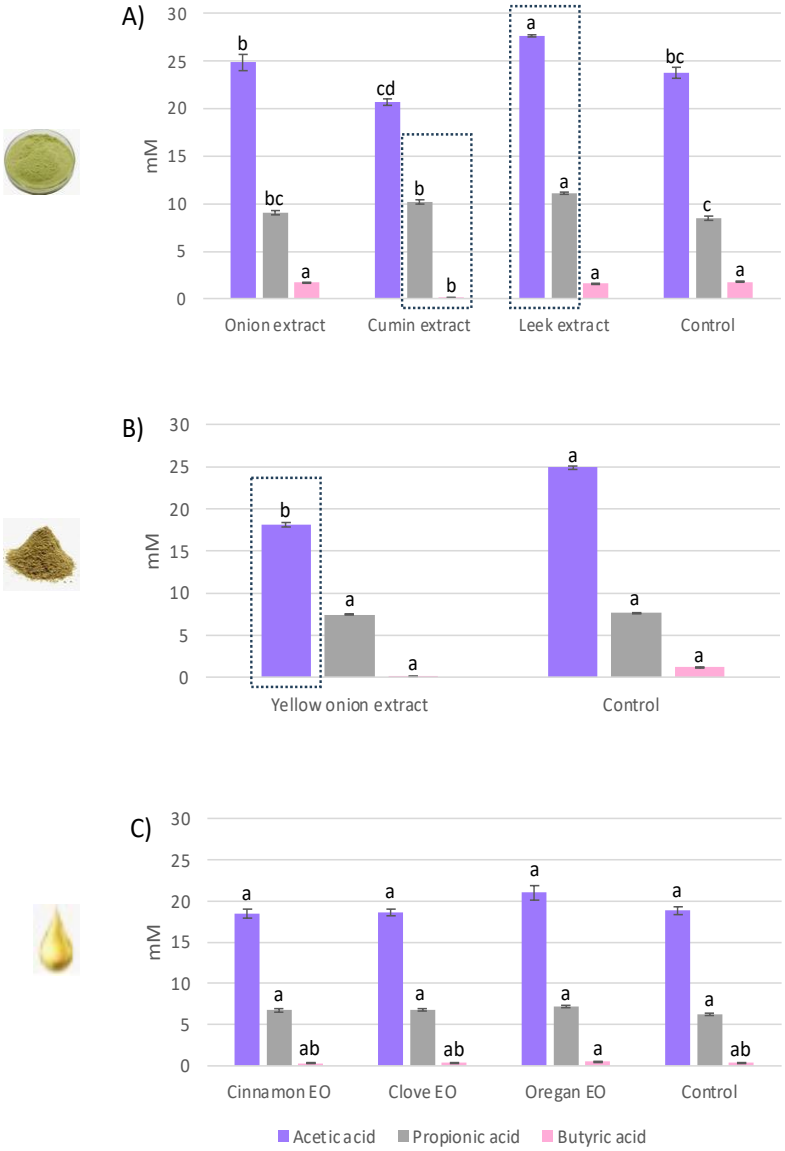

Figure 7. SCFAs concentration (acetic, propionic and butyric acids, mM) at the end point of colonic fermentations (48 h) of DOMCA extracts (P8) (A), yellow onion extract (P1) (B) and essential oils (P4) (C). Different lower-case letters denote significant differences between study products and the control for each fatty acid. For ease of understanding, significant changes have been marked with a dotted box.

On the other hand, Yellow onion extract significantly decreased the production of acetic acid (**Figure 7 B**) and isovaleric acid (results not shown) at the end of colonic fermentation. Finally, Essentials oils did not generate significant changes in SCFAs production (**Figure 7C**).

2.2.2.2. Proteolytic activity of colonic microbiota

The proteolytic activity of colonic microbiota was assessed in terms of ion ammonium production. As expected, ammonium production gradually increased throughout the fermentation process because of progressive depletion of the carbon source. In particular, at 24 h of fermentation a significant lower ammonium ion production was observed with all DOMCA and Yellow onion extracts compared to the control (results not shown). As shown in **Figure 8**, this same pattern was maintained at 48 h of incubation for the cumin and Yellow onion extracts. The lower proteolytic activity of the extracts could be due to the higher availability of carbon source, which would reduce protein metabolism.

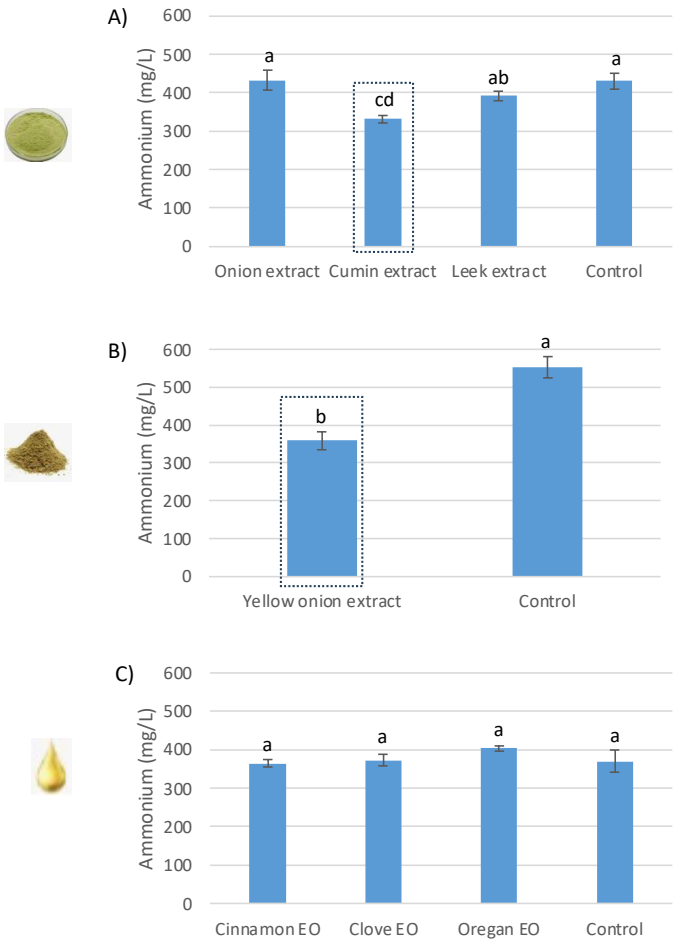

Figure 8. Ammonium production (mg/L) at the end point of colonic fermentations (48 h) of DOMCA extracts (P8) (A), yellow onion extract (P1) (B) and essential oils (P4)(C). Different lower-case letters denote significant differences between study products and the control. For ease of understanding, significant changes have been marked with a dotted box.

2.2.2.3. Effects of microbial-derived metabolites from Yellow onion extract in vitro digestion on an intestinal barrier model

Taking into account all of the above results, the Yellow onion extract that has shown the best gastrointestinal aptitude has been selected to carry out the experiments in the cell model. To this end, we have worked with a cell model that allows us to evaluate the effect of the digests of the ingredients under study on the integrity of the intestinal membrane (Zorraquín-Peña et al., 2021). For this purpose, the cells are initially exposed to a stimulus of intestinal inflammation and under these simulated conditions the effects of our products are evaluated.

The results show that the inflammation cocktail significantly reduced the integrity of the intestinal barrier model at 24h. At 48 h, the chronic inflammation model is observed to slightly improve its integrity due to the absence of inflammatory stimuli, in fact, this is more evident in the case of the control and Yellow onion extract treatments, possibly due to the contribution of proteins from simulation fluids. In any case, there is no difference in the restoration of the model between Onion extract and control (**Figure 9**). In summary, these results suggest that Yellow onion extract did not promote changes in the intestinal permeability, probably due to the few changes observed in the profiles of gut microbiota metabolites (SCFAs, **Figure 7B**) during the simulation. Although no direct *cell-in vitro* effects are exherted, Yellow onion functionality could have health associated effects on a barrier model once the functionalized ingredient intake is able modulation the gut microbiome in a more solid and representative manner. Further studies in other human cells models and targets would be needed for confirming these evidences.

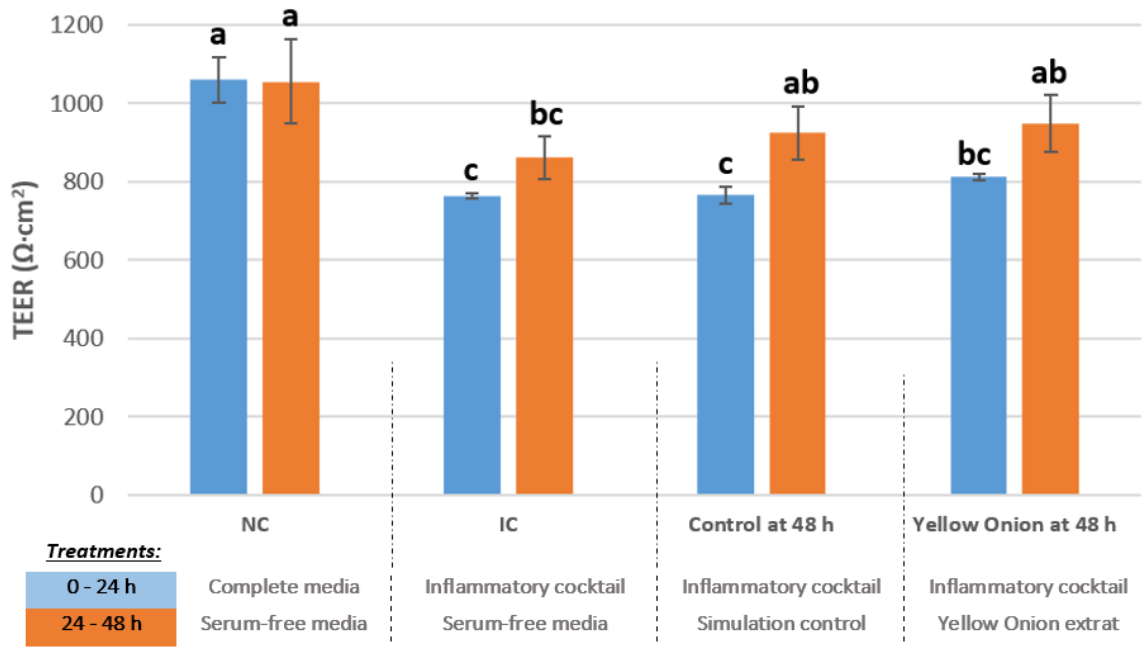

Figure 9. Transepithelial resistance (TEER) of the differentiated Caco-2 barrier cell model exposed during the first 24 h to an inflammatory cocktail (NC: negative control, non-exposed; IC: inflammatory cocktail). During 24-48h, inflamed barrier model was exposed to control digesta (Control at 48h) and yellow onion digesta (Yellow Onion at 48h). Legend bellow shows treatments exposed to different conditions from 0 to 48 h. Different lower-case letters denote significant differences between study products and the control.

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