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Enrichment or depletion? The impact of stool pretreatment on metaproteomic characterization of the human gut microbiota

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Abbreviations: **DC**, differential centrifugation; **FASP**, filter aided sample preparation; **GO-BP**, Gene Ontology biological process; **IU-PF**, InterPro/UniProtKB protein family; **KEGG**, Kyoto Encyclopedia of Genes and Genomes; **KOG**, KEGG orthology group; **LCA**, lowest common ancestor; **LDA**, linear discriminant analysis; **NC**, not centrifuged; **NSAF**, normalized spectral abundance factor; **PSM**, peptide-spectrum match; **TMD**, transmembrane domain.

Keywords: differential centrifugation; gut microbiome; host; metaproteomics; sample preparation.

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1 **Abstract**

2 Up to date, most metaproteomic studies of the gut microbiota employ stool sample pretreatment
3 methods to enrich for microbial components. However, a specific investigation aimed at assessing
4 if, how and to what extent this may impact on the final taxonomic and functional results is still
5 lacking.

6 Here, stool replicates were either pretreated by differential centrifugation (DC) or not centrifuged
7 (NC). Protein extracts were then processed by filter-aided sample preparation, single-run LC and
8 high-resolution MS, and the metaproteomic data were compared by spectral counting. DC led to a
9 higher number of identifications, a significantly richer microbial diversity, as well as to reduced
10 information on the non-microbial components (host and food) when compared to NC. Nevertheless,
11 dramatic differences in the relative abundance of several gut microbial taxa were also observed,
12 including a significant change in the Firmicutes/Bacteroidetes ratio. Furthermore, some important
13 microbial functional categories, including cell surface enzymes, membrane-associate proteins,
14 extracellular proteins, and flagella, were significant reduced after DC.

15 In conclusion, this work underlines that a critical evaluation is needed when selecting the
16 appropriate stool sample processing protocol in the context of a metaproteomic study, depending on
17 the specific target to which the research is aimed.

18

1 **1 Introduction**

2 The human gut harbors a complex microbial community, which is responsible for several key
3 physiological functions of the host, including food digestion, provision of substrates to the gut
4 epithelial cells, and immune responses [1-3]. Moreover, a growing amount of data suggests that
5 changes in the microbiota structure and activity are tightly related to the development of metabolic
6 dysfunctions, allergies, chronic inflammatory diseases, autoimmune disorders and tumors [4-7].
7 Therefore, uncovering the taxonomic composition and functional capacity within the mammalian
8 gut microbiota can provide fundamental information concerning host health and disease. To this
9 extent, metaproteomics grants the unique ability to determine which functions are actually being
10 changed within the gut microbiota depending on the host genetics or environmental factors [8].
11 Several papers have been published so far describing the application of the shotgun metaproteomic
12 approach to stool samples collected from human individuals or animal models with the aim of
13 studying the gut microbiota [9, 10]. In most cases, stool samples have been subjected to enrichment
14 methods (usually by differential centrifugation or related procedures, such as ultracentrifugation
15 using a density gradient medium), in order to remove host cells, undigested food and other debris,
16 and thus to enlarge the dynamic range of microbial protein identifications [11-19]. Conversely, a
17 more conservative, “direct” procedure (i.e. not including an enrichment step) has also been used
18 with success in very few cases [20]. Nevertheless, a specific investigation aimed at elucidating if,
19 how and to what extent sample enrichment steps may impact on the final outcome is still lacking.
20 Here, we evaluated the influence exerted by the differential centrifugation of stool on human gut
21 metaproteomic profiling, using a non-centrifuged, directly extracted, sample as a control. Overall
22 performance, technical reproducibility, as well as taxonomic and functional distribution of the
23 identified proteins were investigated. The consequences of sample pretreatment on information
24 concerning microbiota and host proteomes are discussed.

1 **2 Materials and methods**

2

3 **2.1 Stool sample**

4 The human feces used for this study were provided by a healthy volunteer who gave consent to their
5 use for research purposes. Feces were split into ten samples (as illustrated in **Fig. 1**, top): five
6 (average wet weight 337 mg) were directly subjected to protein extraction, while the remaining five
7 (average wet weight 1,191 mg) underwent differential centrifugation (see below).

8

9 **2.2 Differential centrifugation**

10 Stool samples were subjected to differential centrifugation to enrich for microbial cells, according
11 to VerBerkmoes *et al.* [11] and Tanca *et al.* [21], with minor modifications (see illustration in **Fig.**
12 **1**, bottom). Briefly, samples were resuspended in PBS to reach a final volume of 50 ml, vortexed,
13 shaken in a tube rotator for 45 min, and subjected to low-speed centrifugation at 500 x *g* for 5 min
14 aimed to eliminate particulate and insoluble material. The supernatants were then carefully
15 transferred to a clean polyallomer centrifuge bottle (Beckman Coulter, Brea, CA, USA) and kept at
16 4°C, whereas the pellets were suspended again in PBS. The entire procedure was repeated for a
17 total of three rounds. Finally, the supernatants (one per round, therefore three per sample) were
18 centrifuged at 20,000 x *g* for 15 min, and the derivative pellets were subjected to protein extraction
19 following the protocol described below.

20

21 **2.3 Protein extraction, digestion and quantification**

22 Samples were resuspended by vortexing in extraction buffer (2% SDS, 100 mM DTT, 20 mM Tris-
23 HCl pH 8.8) pre-heated at 95°C. Specifically, a 1:2 (mg/μl) sample-to-buffer ratio was used for the
24 stool samples subjected to direct extraction, whereas the three microbial pellets per sample obtained
25 upon differential centrifugation were first resuspended in the extraction buffer (1:1 ratio) and then
26 pooled, in order to obtain a single tube per sample. Samples were then heated and subjected to a

1 combination of bead-beating and freeze-thawing steps as detailed elsewhere [21]. The protein
2 extract concentration was estimated by whole lane densitometry using QuantityOne software (Bio-
3 Rad, Hercules, CA, USA) after electrophoretic separation through an Any kD Mini-PROTEAN
4 TGX Gel (Bio-Rad) and gel staining with SimplyBlue SafeStain (Invitrogen, Carlsbad, CA, USA).
5 Protein extracts were subjected to on-filter reduction, alkylation, and trypsin digestion according to
6 the filter-aided sample preparation (FASP) protocol [22], with slight modifications detailed
7 elsewhere [23] and using Amicon Ultra-0.5 centrifugal filter units with Ultracel-10 membrane
8 (Millipore, Billerica, MA, USA). Peptide mixtures concentration was estimated by measuring
9 absorbance at 280 nm with a NanoDrop 2000 spectrophotometer (Thermo Scientific, San Jose, CA,
10 USA), using dilutions of the MassPREP *E. coli* Digest Standard (Waters, Milford, MA, USA) to
11 generate a calibration curve.

12

13 **2.4 LC-MS/MS analysis**

14 LC-MS/MS analysis was carried out using an LTQ-Orbitrap Velos mass spectrometer (Thermo
15 Scientific) interfaced with an UltiMate 3000 RSLCnano LC system (Thermo Scientific). The
16 single-run 1D LC peptide separation was performed as previously described [21, 24], loading 4 µg
17 of peptide mixture per each sample, and the mass spectrometer was set up in a data dependent
18 MS/MS mode, with Higher Energy Collision Dissociation as the fragmentation method, as
19 illustrated elsewhere [23].

20

21 **2.5 Data analysis**

22 Peptide identification was performed using Proteome Discoverer (version 1.4.1; Thermo Scientific),
23 with a workflow consisting of the following nodes (and respective parameters): Spectrum Selector
24 for spectra pre-processing (precursor mass range: 350-5,000 Da; S/N Threshold: 1.5), Sequest-HT
25 as search engine (Protein Database: see below; Enzyme: Trypsin; Max. missed cleavage sites: 2;
26 Peptide length range 5-50 amino acids; Max. Delta Cn: 0.05; Precursor mass tolerance: 10 ppm;

1 Fragment mass tolerance: 0.02 Da; Static modification: cysteine carbamidomethylation; Dynamic
2 modification: methionine oxidation), and Percolator for peptide validation (FDR < 1% based on
3 peptide q-value). Results were filtered in order to keep only rank 1 peptides, and protein grouping
4 was allowed according to the maximum parsimony principle.

5 The protein database was generated based on taxonomic information following an iterative
6 approach, as proposed in a recent paper from our group [25]. Specifically, a preliminary search was
7 performed against the complete UniProtKB database (release 2013_12). Then, the peptide
8 sequences identified in all the samples through the preliminary search were uploaded into the
9 Unipept web application (v.2.4, <http://unipept.ugent.be>) [26] to carry out a taxonomic assignment
10 based on the lowest common ancestor (LCA) approach. In keeping with this, sequences from 298
11 detected microbial genera (from Archaea, Bacteria and Fungi; see **Supporting information S1** for
12 details) retrieved from UniProtKB (release 2013_12) were appended to the *Homo sapiens*
13 sequences retrieved from SwissProt (release 2013_12) in order to generate a customized “host-
14 microbiome” database containing sequences from specific microbial taxa and the host (5,990,075
15 protein sequences in total). Furthermore, an additional search was carried out using a “food”
16 database containing all UniProtKB sequences belonging to the 6 most abundant plant genera
17 detected in the preliminary search (namely, *Arachis*, *Musa*, *Corylus*, *Theobroma*, *Glycine* and
18 *Pisum*; 117,047 total protein sequences), and the results were merged to those obtained with the
19 “host-microbiome” database.

20 The Normalized Spectral Abundance Factor (NSAF) was calculated as described elsewhere [24,
21 27], and used in order to estimate peptide abundance. The relative abundance of a feature (protein,
22 taxon, functional categories or combined taxonomic-functional feature) was calculated by summing
23 the NSAF values of all peptides matched to that given feature. The NSAF log ratio was calculated
24 as previously described [28] using 2 as correction factor, and employed to estimate the extent of
25 differential abundance between the two pretreatment methods compared. Statistical significance of

1 differential expression was assessed by applying a t-test on logarithmic NSAF values, after
2 replacing missing values with 0.01 (empirically determined as in [27]).
3 Reproducibility among replicates was measured according to the Pearson correlation coefficient (r),
4 as described elsewhere [29]. Pearson correlation coefficient was calculated by plotting the NSAF
5 values measured for each feature in two different replicates of the same method, and then by
6 calculating the mean values among all possible replicate combinations.
7 Alpha-diversity indexes were calculated according to established methods [30]. InterPro protein
8 families [31] were retrieved from UniProtKB [32]. KEGG orthology groups (KOGs) information
9 [33] was gathered using KOBAS (<http://kobas.cbi.pku.edu.cn/home.do>) [34]. LefSe was used for
10 Linear Discriminant Analysis (LDA) and generation of cladograms
11 (<http://huttenhower.sph.harvard.edu/galaxy/root>) [35], considering features with log LDA score > 2
12 and alpha-value < 0.05 as differentially abundant between sample groups. Protein subcellular
13 localization was predicted using Psort (v.3.0.2, <http://www.psort.org/psortb/index.html>) [36]. The
14 number of transmembrane domains within protein sequences was predicted using the TMHMM
15 Server (v.2.0, <http://www.cbs.dtu.dk/services/TMHMM>) [37]. Data were parsed using in-house
16 scripts, and graphs were generated using Microsoft Excel.
17 The MS proteomics data in this paper have been deposited in the ProteomeXchange Consortium
18 (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository [38, 39] with the
19 dataset identifier PXD001573.

20

1 **3 Results and discussion**

2

3 **3.1 Overall comparison of performance, reproducibility and information depth**

4 In quantitative terms, the samples processed without differential centrifugation (NC) gave a mean
5 protein extraction yield estimated in 26 ± 3 μg of proteins per mg of feces, versus 7 ± 0.5 μg of
6 proteins per mg of feces for the samples enriched by differential centrifugation (DC). In the latter
7 case, the lower protein yield should be due to the fact that most of the proteins contained in the
8 insoluble debris (produced after the first 500 x g centrifugation step) and in the final supernatant
9 (produced after the three sequential 20,000 x g centrifugation rounds) are removed from the sample,
10 and only the final microbial pellet is subjected to protein extraction. Nevertheless, this issue might
11 be relevant only when limited amounts of sample should be available, which is usually not a
12 problem when dealing with human samples.

13 In order to compare the two procedures in qualitative terms, which was the purpose of this work,
14 the same amount of peptide mixture was loaded in the LC column for MS analysis for each sample
15 and condition. As a result, a total of 10,536 and 12,418 non-redundant peptides were identified in
16 the NC and DC samples, respectively (18% increase in DC; histogram in **Fig. 2A**); similar
17 increments were obtained when considering the number of proteins (3,911 for NC versus 4,587 for
18 DC, **Supporting information S2**) or peptide-spectrum matches (PSMs; 69,218 for NC versus
19 81,145 for DC, **Supporting information S3**). Moreover, the percentage of MS/MS spectra reliably
20 matched with peptide sequences was also higher in DC (**Supporting information S4**). Therefore,
21 the DC protocol produces a general increase in the number of identifications.

22 The reproducibility of the two pretreatment methods was also evaluated using the Pearson
23 correlation coefficient (r) as a measure of quantitative reproducibility among replicates. NC and DC
24 exhibited almost identical r values (0.79 for peptides and 0.93 for proteins). Run repeatability in
25 similar experimental conditions was measured and described previously (0.87 for peptides and 0.97
26 for proteins) [21].

1 An LCA approach was used to assign peptide sequences to specific microbial and non-microbial
2 taxa. Accordingly, features unambiguously assigned to a microbial (super)kingdom (Archaea,
3 Bacteria, Fungi) were considered as “microbial”, whereas the other eukaryotic sequences assigned
4 to the phyla Streptophyta (vegetables) or Chordata (host cells and meat) were considered as “non-
5 microbial”. According to the taxonomic classification, the percentage of microbial peptides out of
6 the total was measured as 80% for NC versus 89% for DC (8,401 versus 11,114 in absolute terms,
7 respectively; histogram in **Fig. 2A**). Conversely, the number of non-microbial peptides was over 2-
8 fold higher in NC than in DC (1,458 vs 652, corresponding to 13.8% and 5.3% of the total,
9 respectively; **Supporting information S5**); among them, peptide sequences of plant (food) origin
10 were over 4-fold higher in NC compared to DC (438 vs 104, respectively), while those from the
11 host were about 1.5-fold higher in NC compared to DC (461 vs 302, respectively). These results are
12 consistent with the DC protocol aim of enriching the microbial component by removing host cells,
13 undigested food, fibers, mucus, non-soluble host proteins and complexes in the first rounds of low-
14 speed centrifugation. In keeping with this, the increase seen in the number of identified peptides for
15 the DC protocol is likely due to an enrichment in the microbial component versus other host and
16 food proteins, while the increase in the matched spectra is probably dependent on the reduction of
17 interfering non-protein molecules. Nevertheless, a selective increase in DC of microbial species
18 with better annotated genomic databases might also be a contributing factor, together with the
19 concurrent depletion in proteins from heterogeneous and minor proteinaceous sources from the diet.
20 The cumulative number of microbial and non-microbial peptides detected in five replicate analyses
21 was then calculated, along with the number of identifications common to all replicates (‘core’) (line
22 graphs in **Fig. 2B** and **2C**). Microbial identifications (both ‘core’ and cumulative values) were
23 clearly higher in DC when compared to NC, whereas non-microbial identifications followed the
24 opposite trend. It is interesting to notice that by analyzing a number of $N > 2$ NC replicates it is
25 possible to reach a number of microbial identifications similar to that of a single DC replicate; on
26 the contrary, the number of non-microbial identifications obtained with 5 DC replicates does not

1 reach that achieved with a single NC replicate. Nevertheless, at equal numbers of identified proteins
2 the informative content in terms of microbial diversity (i.e. taxonomy and functions) might still be
3 different for the two approaches (see below). The distribution of the core peptides between NC and
4 DC dataset is shown in the Venn diagrams in **Fig. 2B** (microbial) and **2C** (non-microbial). Over
5 1,200 microbial peptides were consistently detected along all replicates with both methods, while
6 74 and 190 microbial peptides were unique (i.e. found in all replicates of a method and completely
7 undetected with the other method) to NC and DC. Concerning non-microbial peptides, NC provided
8 a dramatically higher contribution in terms of unique peptides when compared to DC (160 vs 4,
9 respectively).

10

11 **3.2 Taxonomic distribution of the gut metaproteome**

12 Almost 80% of the overall microbial peptide sequences were assigned according to an LCA
13 approach to a specific phylum and slightly more than a half to a specific family. Moreover, when
14 comparing NC and DC datasets, no significant variations could be found in the relative amount of
15 microbial peptide sequences assigned to a specific taxon (from the phylum to the genus level),
16 while a slight but statistically significant difference was seen at the species level ($p < 0.05$;
17 **Supporting information S6**).

18 The “metaproteomic alpha-diversity” was also measured, according to the Simpson and the
19 Shannon-Wiener indexes and using taxonomic family abundances as input data. In both cases, DC
20 showed a much higher diversity when compared to NC ($p < 0.0001$; **Supporting information S7**).
21 NC and DC results were also compared based on the relative abundance of the main phyla,
22 according to metaproteomic NSAF data (**Fig. 3A**). Statistically significant differences were
23 observed for all microbial phyla with an abundance higher than 0.1%. In particular, a marked
24 change in the Firmicutes/Bacteroidetes ratio (1.2 for NC versus 2 for DC) was seen, along with a
25 general increase in the relative abundance of the main phyla in DC when compared to NC (e.g., a
26 two-fold increment for Actinobacteria).

1 At the broadest taxonomic level, Firmicutes and Bacteroidetes dominate the gut microbiota in
2 humans and other animals. A lower abundance in Firmicutes has been observed to match with a
3 corresponding increase in Bacteroidetes and vice versa. These phyla include the most abundant
4 variety of bacterial species colonizing the intestine, and a change in their relative abundance has
5 been correlated with a number of metabolic and immunological disorders [40-42]. Therefore, the
6 ability of a method to reliably assess the Firmicutes/Bacteroidetes ratio is crucial, and it should be
7 given careful consideration.

8 **Fig. 3B** shows the comparison carried out at the family level. Many of the main Firmicutes families
9 were significantly enriched in DC, apart from Clostridiaceae (same percentage as in NC) and
10 Oscillospiraceae (significantly higher in NC); conversely, within Bacteroidetes, Bacteroidaceae
11 were much higher in NC, whereas Prevotellaceae exhibited the opposite trend. Of note, among
12 families belonging to the less abundant phyla, Desulfovibrionaceae, Bifidobacteriaceae and
13 Sutterellaceae were enriched almost seven-, three- and two-fold in DC when compared to NC.
14 Therefore, despite the higher alpha-diversity recorded in DC samples, possibly due to a more
15 efficient extraction of microbial proteins when undigested food and host components are depleted,
16 species belonging to Bacteroidaceae and Oscillospiraceae might partition preferentially to the
17 “debris” pellet (see **Fig.1**). The cumulative and ‘core’ number of taxonomic families identified in
18 five replicate analyses are given in **Supporting information S8**. Interestingly, no taxonomic
19 families were found to be present in all DC replicates and in none of NC replicates, and vice versa.
20 Differential NSAF abundances of taxonomic data were also assessed by carrying out a Linear
21 Discriminant Analysis using LEfSe to determine the effect size and to account for the hierarchical
22 structure of the taxonomic ranks. The cladogram in **Fig. 4** depicts the hierarchical relationships
23 between the taxa identified in this study; taxa significantly varying between NC and DC (log LDA
24 score > 2 and alpha-value < 0.05) are presented in color. As apparent from the image, each
25 pretreatment method presents differential trends consistently covering the entire taxonomy tree;
26 examples of ‘class-to-genus axes’ are Bacteroidia-*Bacteroides* (represented by many different

1 species) significantly higher in NC, as well as, among those enriched in DC, Clostridia-
2 *Faecalibacterium*, Actinobacteria-*Bifidobacterium*, Betaproteobacteria-*Sutterella*,
3 Deltaproteobacteria-*Desulfovibrio* and Methanobacteria-*Methanobrevibacter*. Interestingly,
4 however, *Prevotella* (Bacteroidia) and *Ruminococcus* (Clostridia) abundances follow an opposite
5 trend with respect to the related taxa of the same class. To this extent, the most abundant
6 *Ruminococcus* species detected in this study, *R. bromii*, has been previously recognized as
7 specialized to degrade cellulose and to bind tightly and directly to insoluble starch particles in fecal
8 samples [43]. Thus, as considered above, the differential depletion of the Clostridia member *R.*
9 *bromii* (log LDA score > 3 and alpha-value < 0.01) into the discarded pellet might depend on its
10 differential substrate colonization in respect to other members of this class. In addition, while most
11 of the identified species of *Bacteroides* appear to be markedly depleted in DC, two of them (namely
12 *B. massiliensis* and *B. cellulosilyticus*) show a higher abundance in DC (log LDA score > 3 and
13 alpha-value < 0.01 for both). A possible explanation for such “species-dependent”
14 enrichment/depletion of *Bacteroides* in DC samples might be provided by their species-specific
15 colonization “geography” within the host gut environment [44].
16 Additional information concerning differentially abundant microbial genera and species is provided
17 in **Supporting information S9** and **S10**.

18

19 **3.3 Functional features of the gut metaproteome**

20 In order to infer functional information on the gut metaproteome, each identified protein was
21 classified according to three different annotations: Gene Ontology biological process (GO-BP),
22 InterPro/UniProtKB protein family (IU-PF), KEGG orthology groups (KOG). Diverse annotation
23 methods were employed since no consensus exists on the best functional annotation approach for
24 microbiome analysis. Moreover, the investigation of complementary levels of annotation can
25 contribute to enlarge the information depth of a metaproteomic study, especially considering that
26 databases used for peptide identification usually contain many poorly annotated sequences [45, 46].

1 According to the GO-BP classification, a total of 640 and 730 microbial biological process
2 categories were found in NC and DC, respectively (**Supporting information S11**). **Fig. 5A**
3 illustrates the most abundant GO-BP categories. Among those with abundance > 1%, comparable
4 percentages could be observed in most cases for NC and DC, with slight but significant differences,
5 for instance, for translation and transporter activity (higher in NC), as well as for glycolytic process
6 and kinase activity (higher in DC), among others. In addition, 23 categories were found to be
7 significantly differential between NC and DC (log ratio > 1 and *p*-value < 0.01; **Supporting**
8 **information S12**), including proteins related to cell replication and biosynthesis (higher in DC), as
9 well as to pathogenesis and substrate degradation activities (higher in NC, comprising sialidases,
10 collagenases, endopeptidases and other proteins involved in nutrient degradation).

11 Taking into account the known functional redundancy among even unrelated taxa [44], these GO-
12 BP functional categories were combined with taxonomic information, in order to assess the taxa
13 specific contribution and thus to verify whether any of the GO-BP trends was independent from the
14 taxonomic trends described in the previous paragraph. As shown in **Fig. 5B**, for each of the top 12
15 GO-BP categories, the abundance values corresponding to the two main phyla (Firmicutes and
16 Bacteroidetes) were investigated. As a result, proteins assigned to Firmicutes and related to
17 flagellum-dependent motility, amino acid metabolism and polysaccharide catabolism were higher in
18 NC, in clear contrast with the above mentioned taxonomic trend. In the other cases, the differences
19 in abundance are quite consistent with the taxonomic trend. **Supporting information S13-S15**
20 report the most abundant and differential features combining GO-BP information with
21 phylum/family taxonomic assignment.

22 According to the IU-PF classification, a total of 299 and 338 microbial protein families were found
23 in NC and DC, respectively (**Supporting information S16**). **Supporting information S17 and S18**
24 illustrate the most abundant protein families, and those significantly differential between NC and
25 DC, respectively, while **Supporting information S19-S22** show the data concerning the IU-PF
26 functional classes combined with taxonomic assignments. This analysis revealed that the

1 Bacteroidetes TonB-dependent receptor family was significantly higher in NC, and that the
2 Firmicutes (namely Clostridiaceae) Peptidase S8 was not detectable in DC; conversely, examples of
3 protein families enriched in or unique to DC were histone-like proteins from Firmicutes and sulfate
4 adenylyltransferase from Desulfovibrionaceae, respectively.

5 According to the KOG classification, a total of 598 and 687 microbial protein families were found
6 in NC and DC, respectively (**Supporting information S23**). **Supporting information S24** and **S25**
7 illustrate the most abundant KOGs, and those significantly differential between NC and DC,
8 respectively, while **Supporting information S26-S29** refer to the combined functional-taxonomic
9 classification. Firmicutes flagellins and glutamate dehydrogenases were significantly higher in NC,
10 in opposition to the general taxonomic behavior. Furthermore, lactocepins and pullulanases (both
11 cell surface-associated enzymes, with possible biotechnological applications) from various families
12 belonging to Firmicutes and Actinobacteria were dramatically depleted in DC, once again in spite
13 of the global taxonomic trend. We chose to employ in parallel both IU-PF and KOG annotations
14 since we observed that some of the main functional categories found with the former were
15 completely absent in the latter (e.g. TonB-dependent receptor), and vice versa (e.g. flagellin), as
16 clearly evident when comparing **Supporting information S17** and **S24**).

17 One of the most striking observations that emerged when comparing the two methods, DC and NC,
18 was the significant change in the Firmicutes/Bacteroidetes ratio, mostly due to the marked reduction
19 of Bacteroidaceae in DC. In parallel, the functional classes that were significantly more depleted in
20 DC were those associated with hydrolase and endopeptidase activities (**Supporting information**
21 **S13**), accounted for by enzymes which are mainly devoted to degradation of (food) carbohydrates
22 and proteins, respectively. In addition, the most abundant taxonomic-functional class in the whole
23 microbiota was transporter activity associated with the family Bacteroidaceae (**Supporting**
24 **information S14**). The class Bacteroidetes is known for its role in degradation of undigested food
25 residues, mainly represented by dietary glycans. As a further observation, the protein identities
26 assigned to food components were drastically reduced by the DC treatment [47]. When considering

1 all these results, it can be hypothesized that food-degrading functions might undergo a selective
2 depletion in DC, being eliminated together with the undigested food residues in the course of the
3 first centrifugations aimed to remove insoluble debris from the fecal material, and thus leading to
4 the observed variation in the Firmicutes/Bacteroidetes ratio. In addition, it is known that insoluble
5 substrates are colonized by different subsets of fecal bacteria [43, 48]; their removal may therefore
6 lead to the introduction of biases depending on the specific composition of the stool sample under
7 examination.

8 When considering the structural complexity of the bacterial cell and the different protein
9 localization compartments (cytosolic, membrane associated, supramolecular cell-surface associated,
10 or secreted in the extracellular *milieu*), the effect of DC on the final proteomic profile outcome
11 deserves further specific considerations. Based on localization prediction carried out using Psort
12 (**Supporting information S30**), the DC dataset was found to be slightly enriched in cytoplasmic
13 proteins, as well as depleted in membrane and, to a higher extent (over 30% reduction),
14 extracellular/secreted proteins when compared to the NC dataset (significance $p < 10^{-4}$). Moreover,
15 the investigation of microbial proteins containing one or more transmembrane domains (TMDs)
16 revealed that their presence is significantly higher ($p < 10^{-4}$) in NC samples when compared to DC
17 samples. When considering the abundance distribution of TMD-containing proteins among bacterial
18 phyla, differences were observed in DC vs NC for Firmicutes (6% vs 9%, $p < 10^{-5}$), and
19 Actinobacteria (4% vs 28%, $p < 10^{-5}$), while TMDs from Bacteroidetes (11% vs 11%) and
20 Proteobacteria (13% vs 11%) seemed unaffected by the DC protocol. An important conclusion can
21 be drawn from these observations. When applying the DC protocol, there is the risk for the sample
22 to undergo a selective depletion not only in taxonomic terms (i.e. a general depletion in
23 Bacteroidetes, as noted above), but also in structural terms, as observed here for
24 extracellular/secreted and membrane proteins. In fact, adding to the expected loss of highly soluble,
25 secreted proteins due to removal of the final supernatant, other physico-chemical features may favor
26 a differential partitioning of the proteins along the centrifugation steps. For instance, highly

1 hydrophobic or “sticky” proteins that remain attached to the solid surfaces offered by sloughed cells
2 and undigested food, such as bacterial adhesins or enzymes with substrate-binding and degradation
3 functions, would be removed when eliminating the debris in the first steps of the DC protocol . In
4 the case of Actinobacteria (and especially of *Bifidobacterium*), for example, membrane proteins
5 depleted by the DC treatment were mainly pullulanase and subtilisin-like serine protease, which
6 both have a transmembrane anchorage and a surface-exposed catalytic portion. In addition, a
7 contribution to this bias would be provided also by residual cell wall and membrane fragments from
8 dead bacterial cells. Likewise, in clear contrast with the above mentioned taxonomic trend,
9 Firmicutes flagellins were depleted in DC. As a further consideration, there would also be the
10 possibility of separating bacteria that are actively expressing particular subsets of proteins from
11 those that are not expressing them. Finally, we cannot rule out that proteolytic events or slight
12 changes in protein expression in living microbial cells may occur during the DC process. Therefore,
13 careful scrutiny of this scenario should be given when selecting a sample pretreatment strategy for
14 proteomic characterization of the microbiota.

15

16 **3.4 Functional features of the host proteome**

17 The main aim pursued when employing a DC pretreatment is the removal of host proteins, which
18 are usually considered as contaminants. However, when investigating gut metaproteome changes
19 related to specific physiological or pathological conditions, preservation of host proteome
20 information may be useful to shed light on the concurrent modifications occurring in the gut
21 environment (e.g. intestinal immune response, cell junctions, mucus layer).

22 Therefore, in order to investigate qualitative and quantitative differences between the host
23 information achieved using NC and DC protocols, peptide sequences unambiguously assigned to
24 the order Primates (and thus distinguished from food peptides of other mammalian origin, i.e. from
25 meat) were selected for further analysis concerning the host proteome (peptide identification
26 statistics are shown in **Supporting information S31**). As done for the microbial proteome, host

1 proteins were classified according to GO-BP, IU-PF and KOG annotations, and relative abundances
2 of all functional categories were comparatively assessed for NC and DC (**Supporting information**
3 **S32-S34**). The main results can be summarized as follows: i) human glycosyl hydrolases were
4 found as significantly more abundant in NC, as already observed for the microbial enzymatic
5 counterpart; ii) human serine endopeptidase inhibitors (serpins) were higher in NC consistently with
6 the higher abundance in NC of the microbial serine endopeptidases; iii) several proteins related to
7 functions of considerable biological importance in the gut (including some specific members of
8 MHC class I and II, mucin, antitrypsin, antichymotrypsin e peptidase families) were significantly
9 depleted in the DC dataset; iv) elastase and phospholipase A2 were among protein functions
10 relatively enriched in DC. Taken together, these data highlight, as expected, that the NC protocol
11 may be preferable for studies that aim to gather microbiome data along with corresponding host
12 information.

13

1 **4 Concluding remarks**

2 The results presented in this work highlight pros and cons of the stool pretreatment based on DC
3 with regard to the metaproteomic analysis of the human gut microbiome. Among the advantages,
4 samples processed by DC generally achieve a higher number of protein/peptide identifications, with
5 a significantly higher microbial diversity. This is undoubtedly of key importance when conducting a
6 study aimed at assessing subtle changes in the gut microbiota, as well as at identifying very low
7 abundance enzymes involved in specific microbial pathways. However, the elimination of
8 particulate matter, such as food and mucous residues, heavily colonized by specific assortments of
9 microbial taxa, appears to introduce a clear bias towards “free roaming” microbial cells. In addition
10 to taxonomy, functional and structural information is also affected, when considering the depletion
11 observed in specific functional categories, such as flagella or cell surface anchored enzymes. As a
12 further observation, information on the non-microbial counterpart (host- and food-derived proteins)
13 is dramatically reduced when applying the DC protocol. Finally, it is also worth noting that the DC
14 procedure is considerably more labor intensive and time consuming, and that it may be more
15 influenced than NC by the wide variability in feces texture, fiber and water content. In conclusion,
16 this work clearly underlines that a critical evaluation needs to be made prior to selecting how to
17 process stool samples in the context of a metaproteomic study.

18

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6

7 **Conflict of interest statement**

8 The authors have declared no conflict of interest.

9

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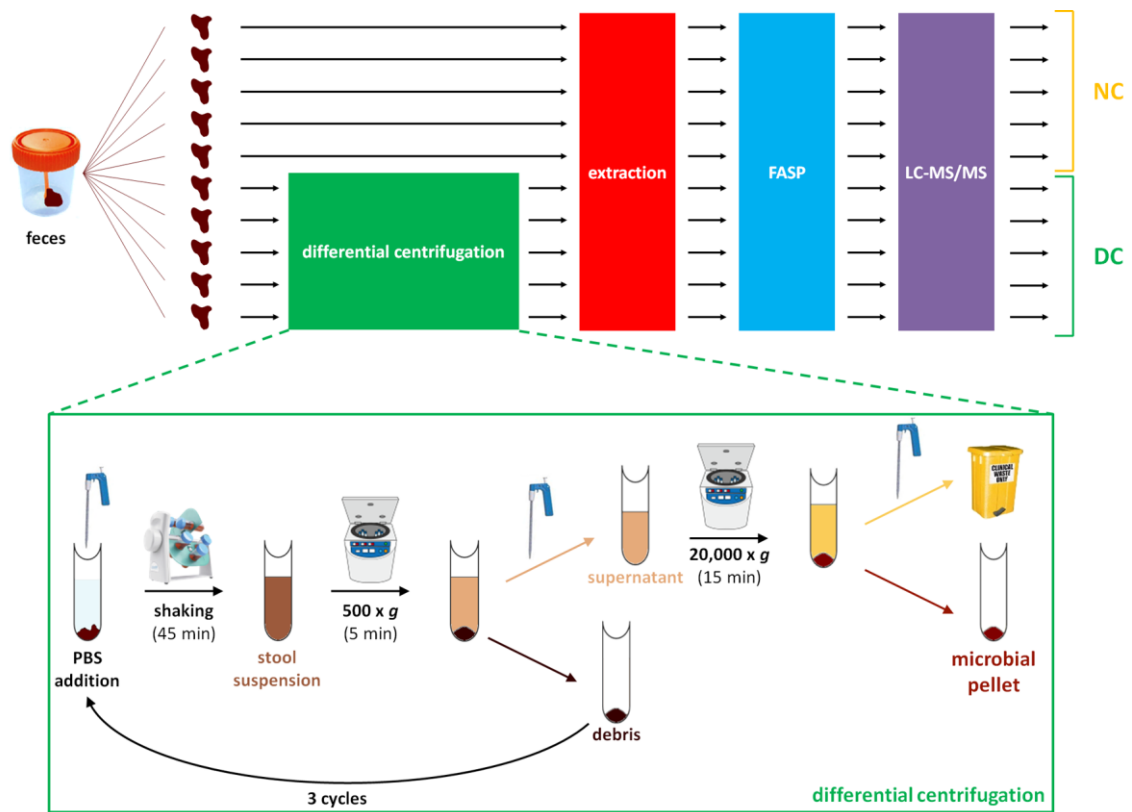
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1 **Figure legends**

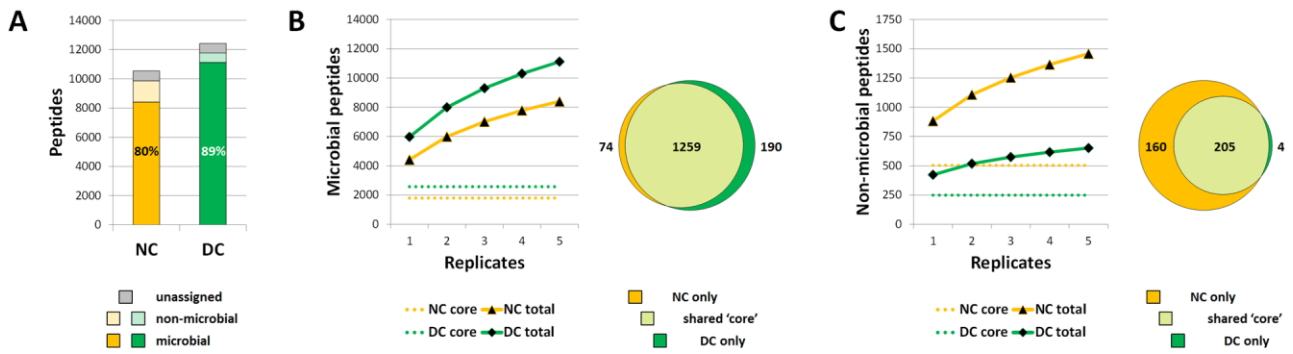
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4 **Figure 1.** Schematic representation of the study design, with detailed description of the
5 experimental steps comprised in the differential centrifugation pretreatment (green box).

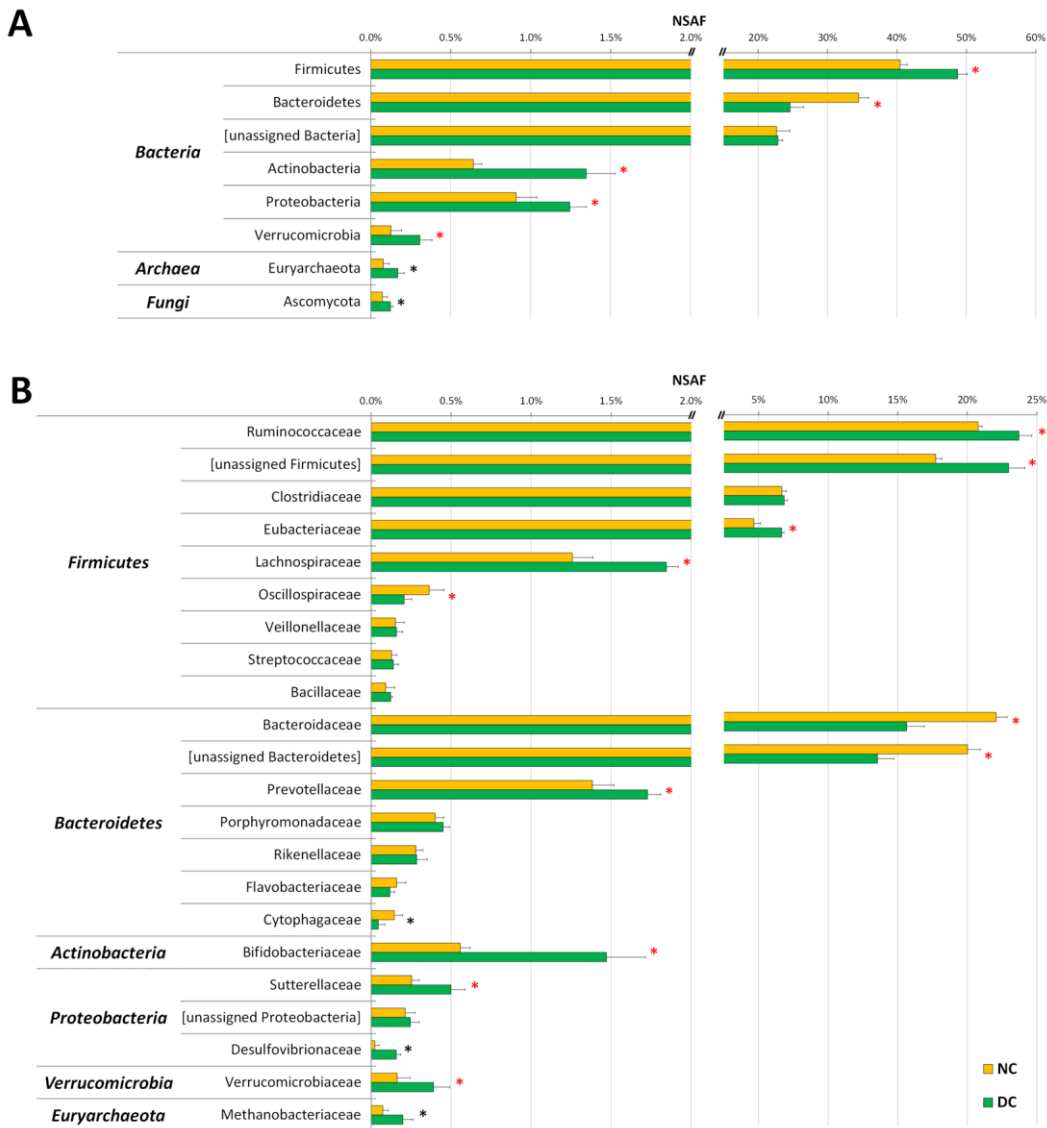
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2 **Figure 2.** Peptide identification statistics. (A) Histogram comparing the total number of peptides
 3 identified without (NC) or with differential centrifugation (DC). Opaque and transparent bars are
 4 referred to microbial and non-microbial peptides, respectively; grey bars represent peptides with
 5 unassigned taxonomy. Percentage values indicate the relative amount of microbial identifications
 6 compared to the total. (B) Left, line graph illustrating the cumulative number of microbial peptides
 7 detected in five replicate analyses (solid lines), along with the ‘core’ identifications (common to all
 8 replicates, dashed lines). Right, Venn diagram depicting the distribution of the microbial ‘core’
 9 peptides in the NC and DC datasets. Specifically, the light green overlapping part comprises
 10 peptides detected in all replicates with both methods, while the orange (or dark green) side refers to
 11 the peptides found in all NC (or DC) replicates and completely undetected in DC (or NC). (C) The
 12 same as in (B), but concerning non-microbial peptides.

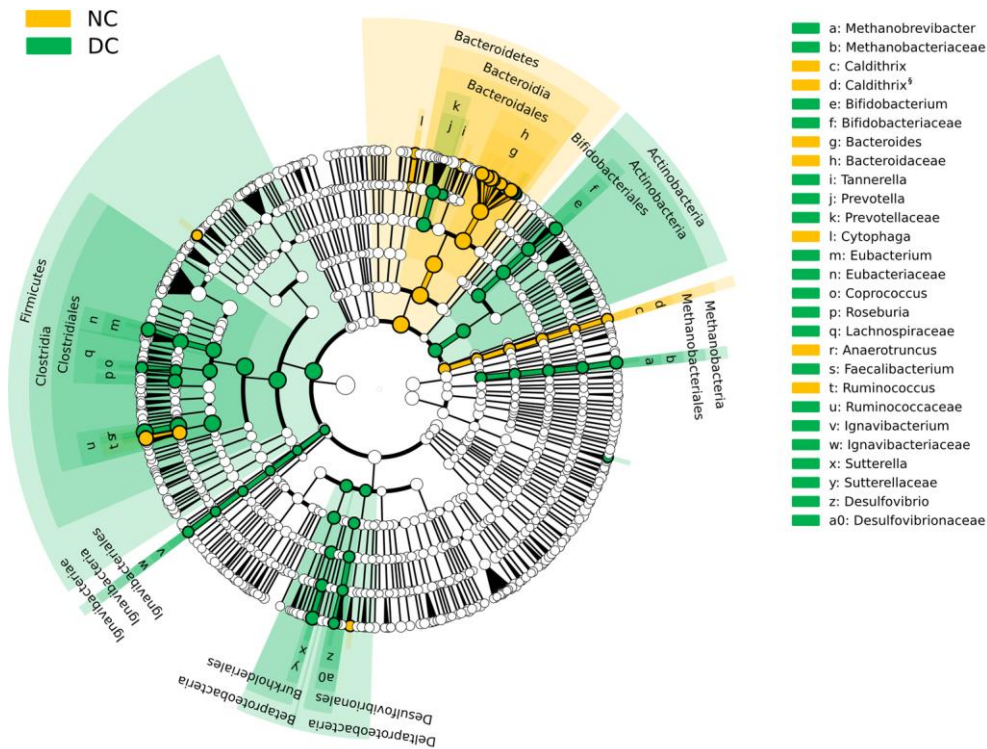
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1

2 **Figure 3.** Bar graphs illustrating the microbial phyla (A) and families (B) with a mean abundance
 3 higher than 0.1% in NC and/or DC. Peptide taxonomic assignments were carried out based on an
 4 LCA approach using Unipept. Phyla (A) and families (B) are grouped based on the (super)kingdom
 5 and phylum to which they belong, respectively. Peptide sequences which could not be assigned to a
 6 specific phylum (A) or family (B) but only to a higher taxonomic level are shown in square brackets
 7 and named as “unassigned” followed by the higher taxonomic level to which they belong. Black
 8 and red asterisks indicate a statistically significant difference between groups with $p < 0.05$ and $p <$
 9 0.01 , respectively.

10



1

2 **Figure 4.** Cladogram showing a hierarchical representation of the taxa identified in this study,
 3 generated based on the LEfSe analysis. Each taxon (from the phylum to the species level) is
 4 represented by a circle whose size is proportional to the highest logarithmic abundance between the
 5 two groups. Taxa with significantly different abundance between NC and DC (Kruskall Wallis
 6 alpha-value < 0.01 and log LDA score > 3) are colored. Phylum, class and order names are reported
 7 within the cladogram, whereas family and genus names are marked with a letter (the legend on the
 8 right reports these letters followed by the corresponding taxon name). [§]Since the family to which
 9 the genus *Caldithrix* belongs is currently unclassified (as well as phylum, class and order), it has
 10 been generically indicated with the same name of the genus.

11



1

2 **Figure 5.** GO-BP classification of the identified proteins. (A) Bar graph illustrating the top 12 GO-
 3 BP categories according to the NSAF abundances of the related proteins. Asterisks indicate a
 4 statistically significant difference between groups ($p < 0.01$). (B) Taxonomic assignment of
 5 functional categories shown in (A): for each GO-BP category, the abundance values corresponding
 6 to the two main phyla (Firmicutes and Bacteroidetes) are reported.