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Fermented food consumption modulates the oral microbiota

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Fermented food consumption is recommended for health and environmental purposes. While it is known to impact gut microbiota, further investigation is needed to establish connections with the oral microbiota. For this purpose, we investigated the effect of daily consumption of a model cheese containing 3 Lactic Acid Bacteria (LAB) species on the oral microbiota of rats following a 3-week diet. Cheese consumption transiently modifies the oral microbiota and leads to a transient persistence of LAB in the oral cavity of 1/3 of the animals. The origin of this variability was partly explained by an overrepresentation of salivary proteins involved in the response to oxidative stress in animals without LAB persistence. These findings highlight the significance of fermented foods in shaping the diversity of the oral microbiota. Additionally, they suggest that variations in the salivary proteome among individuals may influence the permissiveness of the oral microbiota towards exogenous microorganisms.

Fermented foods represent a relevant alternative to face global changes by limiting energy costs during food processing and storage and contributing to human health¹. Hence, fermented foods are sources of living microorganisms, and their regular consumption, in particular the ones containing Lactic Acid bacteria (LAB), is a promoting factor to prevent or overcome gut dysbiosis and other diseases^{2,3}. Together, these properties have increased the demand for fermented food, leading to an anticipated annual market growth of about 6.35% (Compound Annual Growth Rate 2022–2027)⁴. As a result, fermented food has now assumed a substantial role in the human diet⁵.

Although the effect of fermented food consumption on gut physiology is well documented⁵, the effect of their consumption at the entrance of the digestive tract, the oral cavity, has been poorly investigated. However, the massive and regular intake of the LAB that is present in fermented food could have an important impact on the oral environment and particularly the oral microbiota. For instance, the consumption of 100 g of yogurt, which must contain a minimum of 10⁹ live LAB⁶, represents approximately 10% of the total bacteria of the oral microbiota.

The oral cavity, serving as the entry point to the digestive tract, is directly exposed to foodborne microorganisms. Despite its importance, the impact of consuming fermented foods on the oral environment, specifically on the oral microbiota, remains an underexplored area of study. Within the oral cavity resides a complex ecosystem that is not a favourable location for the persistence of exogenous microorganisms, due to the fact that it contains 10¹⁰ bacteria and more than 700 different species that are divided into different

ecological niches⁷. Each of these niches presents unique physico-chemical and biological characteristics, which in association with the lack of free space, can limit the persistence of the less adapted bacteria. In addition, many endogenous bacteria in the oral microbiota secrete antimicrobial peptides directed against exogenous bacteria, thus interfering with their persistence⁸. The saliva that bathes the oral cavity also contains host and bacterial antimicrobial compounds, which can also limit oral bacterial persistence⁹.

Despite the hostile conditions of the host oral cavity, some exogenous bacteria can persist in the oral cavity^{10–12}. Many studies focusing on LAB persistence in the oral cavity have been conducted by using fermented dairy products, such as yogurt, cheese, or fermented milk^{10,11,13,14}. The duration and the amount of persistent exogenous bacteria in these studies were highly variable and did not allow us to draw definitive conclusions¹⁵. Indeed, other factors can explain these differences, as some bacterial strains are better adapted to the oral environment than others^{16,17}; moreover, the fermented food matrix can modulate the physiology of the food bacteria¹⁸. In addition, products with solid matrices, such as pressed cheeses, require longer chewing time to be unstructured before swallowing, thus leading to a longer time for the release of LAB in the oral cavity and thereby facilitating oral persistence¹⁹. Finally, the duration of the diet and the number of ingested bacteria are factors playing an important role in the variability of bacterial persistence^{16,20}.

In a previous study, our group investigated the persistence of 3 LAB strains (*Streptococcus thermophilus*, *Lactococcus lactis*, and

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Lactocaseibacillus paracasei) in the oral cavity of rats after one week of a cheese-based diet¹⁰. The persistence of at least one strain was observed in 1/3 of the animals after the end of the diet. However, the factors that could explain this persistence variability were not investigated. Therefore, this study aimed to explore the impact of fermented food consumption on the oral microbiota and LAB persistence and to investigate the role of the oral environment, including oral microbiota composition and the salivary proteome, on this persistence.

Results

Effect of diet on salivary microbiota and LAB persistence after the three periods

The impact of the diet on salivary microbiota was investigated by 16S sequencing rRNA at the end of the three periods for the three groups. The alpha-diversity (Fig. 1A) in the salivary microbiota was compared between the three groups of rats at the end of each period by using the observed species richness (OSR), as well as, Chao1, Shannon, InvSimpson and Fisher index represented in Supplementary Fig. S2. During the pre-diet period, no significant difference was observed in the mean OSR. At the end of the diet period, the means of OSR for the ICD and CD groups were significantly lower than the mean of the SD group ($p < 0.05$), showing a negative impact of the cheese matrix (ionised or non-ionised) on the OSR. At the end of the post-diet period, the mean OSR for the CD group was significantly lower than the SD ($p < 0.01$) and ICD groups ($p < 0.001$). This result demonstrated

that live LAB caused a more lasting modification of the microbiota richness than the cheese matrix.

The β -diversity analysis (Fig.1B) revealed differences in the microbial community structure (abundance and proportions) between the groups. These differences can be observed at the end of the cheese-diet period with significant separations observed between the CD group and the two other groups ($p < 0.003$ for SD and $p < 0.006$ for the ICD group). At the end of the post-diet period, the SD and ICD groups were mixed, whereas the CD group was significantly separated from these two groups ($p < 0.003$). Taken together, these results indicate that the consumption of cheese with live LAB could modify the composition of the salivary microbiota through additional matrix and LAB effects. The profile of the salivary microbiota at the genus level is presented in Fig. 1C (and at the phylum and family levels in Supplementary Fig. S3). The end of the pre-diet period was characterised by significant differences between the SD group and the two other groups. At the end of the cheese-diet period, most of the significant differences occurred between the CD group and the two other groups, with 8 genera differing in their relative abundance: *Bacteroides*, *Bifidobacterium*, *Corynebacterium*, *Eisenbergiella*, *Enterobacter*, *Enterococcus*, *Faecalibacterium*, and *Lachnospiraceae* UCG-004. The end of the post-diet period was also characterised by many significant differences between the CD group and the two other groups, with 5 genera differing in relative abundance between the CD group and the two other groups: *Agathobacter*, *Bacteroides*, *Blautia*, *Enterococcus*, and *Pseudomonas*, and with 3 genera differing between the

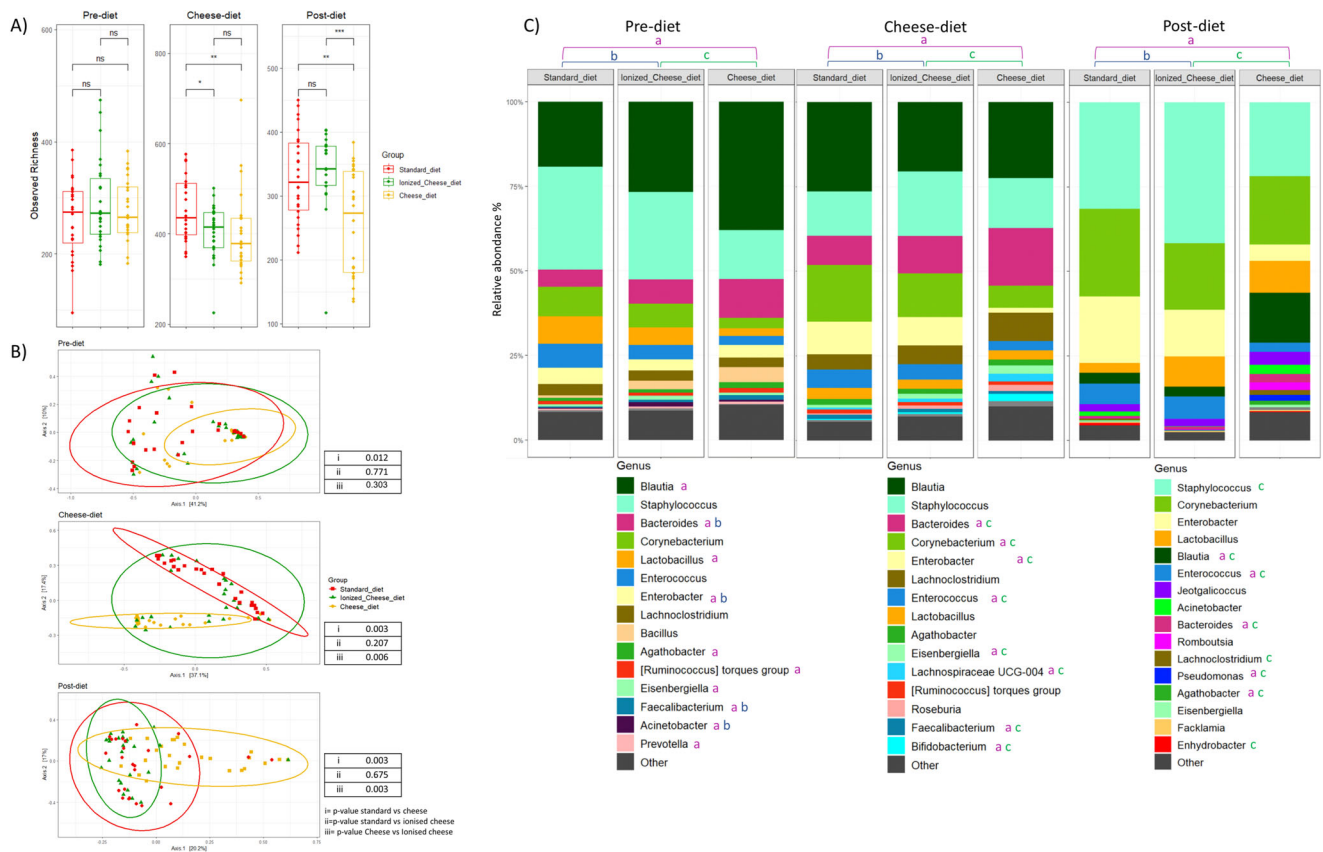


Fig. 1 | Composition of the salivary microbiota at the end of the 3 experimental periods. Composition of the salivary microbiota at the end of the pre-diet, cheese-diet, and post-diet periods by 16 S rRNA sequencing. **A** Alpha diversity is represented by the observed species richness for the standard-diet (red), ionised cheese-diet (green), and cheese-diet (yellow) groups ($n = 27$ /group). Observed species richness values are means \pm SEMs compared via one-way ANOVA followed by a Tukey’s post hoc test. * indicates p value < 0.05 ; ** indicates p value < 0.01 , and *** indicates p value < 0.001 . **B** Principal coordinates analysis (PCoA) multi-dimensional scaling (MDS) of Bray–Curtis dissimilarities (β -diversity) for the

standard-diet (red), ionised cheese-diet (green), and cheese-diet (yellow) groups ($n = 27$ /group). Each point of the PCoA analysis represents one animal. PCoA values were compared with a permanova test. SD, standard diet, CD, cheese diet, and ICD, ionised cheese diet. **C** Average relative abundance of the salivary bacterial genus for each group ($n = 27$ /group) at the end of the three periods. The relative abundances (%) at the genus level were compared by using a Kruskal–Wallis test followed by a Dunn post hoc test. Each letter denotes a significance (p -value < 0.05) of the compared groups: a (pink) = CD vs. SD group, b (blue) = ICD vs. SD group, and c (green) = ICD vs. CD group.

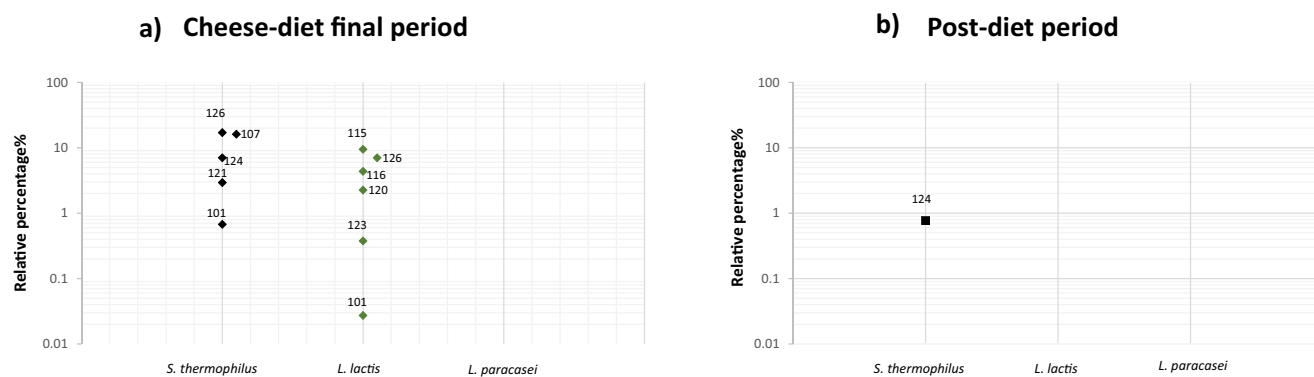


Fig. 2 | Oral persistence of cheese LAB species. The relative percentage of cheese LAB species (*S. thermophilus*, *L. lactis*, and *L. paracasei*) compared to the total bacterial in the oral cavity of the cheese-diet group ($n = 27$) at the end of the cheese-diet period (a) and at the end of the post-diet period (b) by qPCR. Only the CD group

was represented, as cheese LAB was not detectable in the other groups at any of the periods. The corresponding rat numbers are indicated near the dots. The detection threshold was 10–3%.

CD group and the ICD group: *Enhydrobacter*, *Lachnoclostridium*, and *Staphylococcus* (Fig. 1C and Supplementary Fig. S4). These results confirmed the observations that were made at the end of the previous period; specifically, there was an impact of living LAB consumption on the salivary microbiota.

None of the three LAB species were detected in the oral cavity of animals that had never eaten cheese, which validates the specificity of the qPCR. To determine whether cheese LAB species persisted in the oral cavity, samples were collected 23 h after the last cheese intake (Fig. 2a). Only the relative quantification of the CD group after the cheese-diet and post-diet periods are represented, as no bacteria were detected in the other groups and periods. At the end of the cheese-diet period, *S. thermophilus* and *L. lactis* were detected in 5/27 and 6/27 animals of the cheese-diet group, respectively, and it should be noted that *S. thermophilus* and *L. lactis* were both detected in rats 101 and 126. This resulted in 9/27 animals presenting with cheese LAB in their oral cavity. The relative quantification for *S. thermophilus* ranged from 0.676% to 17.1%, whereas it ranged from 0.0273% to 9.47% for *L. lactis*. Moreover, *L. paracasei* was not detected (Fig. 2a). The frequency of LAB detection considerably decreased at the end of the post-diet period since only one animal, rat 124, presented a detectable amount of *S. thermophilus*. It should be noted that this species had already been detected in this animal at the end of the cheese-diet period with a relative abundance 10 times higher than at the end of the post-diet period (Fig. 2b).

Characterisation of the oral environment before exposure to the cheese diet

As described above, cheese bacteria were detected in the oral cavity of 9/27 rats at the end of the cheese-diet period and were not detected in the other 18 animals, even though they all received the same portion of cheese. This variability in the oral persistence of LAB within the CD group is intriguing and deserves further investigation. To explore the phenomena at the origin of this variability, our strategy consisted of exploring the characteristics of the oral environment before exposure to the cheese diet which corresponds to the “pre diet saliva sampling”. This was accomplished via the study of the salivary microbiota and the salivary proteome of the 9 animals presenting a LAB persistence and 9 animals without any LAB detection, which were randomly selected from the CD group.

Before cheese exposure, the salivary microbiota profiles did not differ between animals with and without LAB persistence at the phylum (Fig. 3a) and genus levels (Fig. 3b). Furthermore, no relationship between the alpha and beta diversity and LAB persistence was observed before cheese exposure (Supplementary Fig. S5).

To determine if some salivary proteins are implicated in LAB persistence, the salivary proteomes of the animals exhibiting persistence and

control animals without persistence were analysed at the end of the pre-diet period. Three hundred eighty-three proteins were identified and quantified. Seventy-two proteins were significantly overrepresented (q -value < 0.05) in the animals without LAB persistence; remarkably, none were underrepresented (Table 1). These proteins can be grouped according to their functions, including protein degradation activity (9 proteins), antioxidant and oxidoreductase activities (11), binding activity (15), hydrolase activity (9), lyase activity (5), transferase activity (9), isomerase activity (3) and regulatory and structural activities (4). Interestingly, the protein degradation functions contained almost all of the 20S proteasome subunits with $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, and $\alpha 7$, as well as $\beta 2$, $\beta 3$, and $\beta 5$ with a level, on average, 4 times higher in the animals without LAB persistence. Many proteins involved in antioxidant function and protein repair processes against oxidation were 5 times overrepresented, such as peroxiredoxins, glutathione, thioredoxin, superoxide dismutase, glutaredoxin, heat shock protein, lysozyme C-1, lactogluthione lyase, and glyoxalase domain-containing protein 4. Finally, the proteins from the binding group were, on average, 3.5 times overrepresented and contained mainly proteins from the lipocalin family, including Odorant Binding Proteins and von Ebner gland protein, which are involved in the transport of chemo-sensory molecules.

Interestingly, many of the proteins of the binding family exhibit oxidations with 9 out of 15 modified proteins (Table 1). These modifications included a mass adduct corresponding to oxidation, hydroxylation, and dihydroxylation. Quantification results of the oxidised form (Fig. 4) demonstrated a higher abundance in the saliva of animals without LAB persistence, with Lipocalin 4 and Lipocalin_cytosolic_FA being significantly different.

Discussion

The first objective of this study was to assess whether the regular consumption of fermented food leads to a modulation of the oral microbiota and the persistence of the food bacteria, such as lactic acid bacteria. The modulation of the oral microbiota was observed at the end of the cheese diet when the bacterial richness was lower in the groups exposed to the cheese matrix (ionised or non-ionised)²¹. This effect may result from the presence of antibacterial peptides in the cheese matrix. Some of these antibacterial peptides are produced by the hydrolysis of milk caseins during the cheese ripening period by enzymes such as chymosin or endogenous plasmin, which are naturally present in milk²². The production of these peptides is regulated by different parameters, such as the amount of available casein or the content of salt and fat that is present in cheese. A lower content of salt and fat corresponds to a higher degree of proteolysis²². It is worth mentioning that the cheese used is low in fat (2%), and it is likely to contain a significant amount of bioactive peptides. In addition, some antibacterial peptides may be secreted by the three starters used^{23–25}. Altogether, the presence of these antibacterial peptides can explain the lower richness that

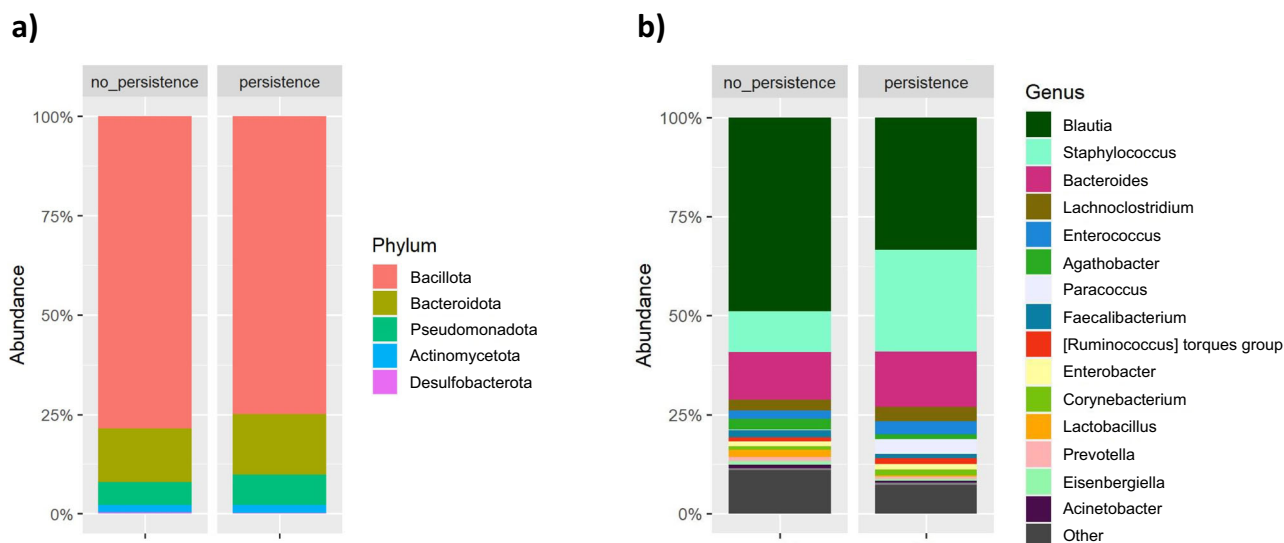


Fig. 3 | Relative abundance of salivary bacterial taxa. Average relative abundance (%) of salivary bacterial phylum (a) and genus (b) for the rats with and without LAB persistence (known as “persistence” and “no persistence”, respectively) at the end of

the pre-diet period ($n = 9/\text{group}$). The relative abundances were compared by using a Kruskal-Wallis test. No significant differences were observed.

was observed at the end of the diet, including in the group of animals fed with ionised cheese, due to the fact that radiation does not affect their bioavailability²⁶. Interestingly, this lower diversity only persisted until the end of the post-diet period in the group that was previously fed with cheese containing live LAB. It is likely that animals eating cheese with live bacteria are exposed to more bioactive peptides than those eating ionised cheese because bacteria continue to produce peptides during cheese storage^{23–25}. Furthermore, while the capacity of LAB to persist in the oral cavity may decrease with time, they might continue to extend the antibacterial effect in the post-diet period by continuously releasing antibacterial peptides. The effect of LAB consumption on oral microbiota diversity is also visible when comparing the microbiota profiles at the family level at the end of the cheese-diet and post-diet periods. These differences may be due to the specific antibacterial effects of LAB on some families or species. For example, *L. lactis* is involved in the secretion of nisin directed against certain Enterobacteriaceae²⁷. In this study, approximately 1/3 of the animals that were exposed to live LAB still had these bacteria in their oral cavity after the cheese-diet period. Interestingly, this occurrence is similar to the one that was previously observed after only one week of a cheese diet¹⁰.

The establishment of oral microbiota is mainly driven by environmental factors²⁸ and this could also be the case for the persistence of food bacteria. However, in this study, the length of exposure to the food and the environmental factors were equally controlled among all the rats, thus eliminating them as possible explanations for the interindividual variability that was observed. The origin of this variability should be sought in the biological factors. This has been performed by studying the characteristics of the oral environment of the animals exhibiting LAB persistence before any exposure to the diet, i.e. the end of the pre-diet period, to find any predisposition to oral LAB persistence. In our conditions, the endogenous oral microbiota does not appear at the origin of the interindividual variability in the persistence of LAB, as the microbial composition at the phylum or family levels was similar. However, the salivary proteome appears to play an important role, with 72 proteins negatively related to the persistence of LAB. A correlation matrix between the oral bacterial composition at the genus level and the overrepresented salivary proteins is provided in Supplementary Fig. S6. Such involvement of the salivary proteome is not surprising because it is known to play an important role in the defence of the oral cavity against pathogens²⁹. The mechanisms that were observed in this study deserve attention since they do not involve proteins whose main activity is

antimicrobial. Remarkably, we found an important representation of 20S proteasome subunits. The proteasome plays a key role in maintaining proteostasis by degrading damaged proteins. There are two main types of proteasomes: the 26S proteasome and the 20S proteasome. The 26S proteasome is composed of the 20S proteasome, which possesses proteolytic activity, and a 19S regulatory unit, which has a function of recognising damaged ubiquitin-tagged proteins³⁰. The absence of the 19S regulatory unit is not surprising because the 20S unit has previously been shown to be one of the most abundant proteasomes in human fluids such as saliva, tears, and sweat³¹. The main role of the 20S proteasome is the removal of abnormal and oxidised proteins and it is considered a “secondary antioxidant defence system”^{32,33}. Several studies have suggested that there is a significant increase in 20S proteasome-associated proteolysis when the cell experiences an increase in oxidative stress^{34,35}.

In this experience, we assume that all animals were exposed to similar levels of oxidation, as they lived in the same environment with identical experimental conditions. However, every individual reacts differently to a threat such as oxidative stress. Here, the animals without bacterial persistence appear to be more sensitive to oxidative stress. This is supported by the fact that most of the proteins with a binding function are oxidized and particularly, lipocalins, including odorant binding proteins (OBPs). Lipocalins play a protective role against oxidative stress in vertebrates by scavenging lipophilic molecules that have been damaged by oxidative stress and that are potentially harmful to the cell^{36,37}. When oxidised, these lipocalins lose their protective function against oxidative stress, which would lead to an increase in the oxidation level. In addition to the proteasome response, animals without LAB persistence appeared to overreact to oxidative stress. The reasons why animals growing in the same environment can exhibit such differences remain to be explained. LAB are known to have a high oxidative stress tolerance, which varies according to the bacterial species and their antioxidant activity^{38,39}. This result would suggest that the absence of LAB species from cheese in the oral cavity of rats is due to a level of oxidative stress incompatible with their persistence.

In conclusion, three weeks of a cheese diet seems to be sufficient to produce transient changes in the diversity of the oral microbiota independently of LAB persistence. Indeed, the persistence of cheese LAB in the oral cavity affects 1/3 of the animals that consumed it. The presence of an oxidative stress response seems to be a key factor in

Table 1 | Salivary proteins overrepresented in 9 rats without cheese LAB persistence at the end of the pre-diet period compared to the 9 rats with LAB persistence

UniProt: accession number	Protein name	Mass (Da)	Anova (p value)	q -value	Fold change	Oxidations
Protein degradation activity						
P40307	Proteasome subunit beta type-2	22912.3	7.49E-05	6.65E-03	5.8	
P17220	Proteasome subunit alpha type-2	25926.5	5.1E-04	2.10E-02	4.4	
P60901	Proteasome subunit alpha type-6	27399.4	8.9E-04	2.33E-02	3.6	
A0A0G2K0 W9	Proteasome subunit alpha type 7	27855.8	9.2E-04	2.33E-02	3.8	
P40112	Proteasome subunit beta type-3	22949.0	1.42E-03	2.45E-02	5.4	
G3V7Q6	Proteasome subunit beta type-5	28559.3	3.92E-03	3.34E-02	3.3	
P18422	Proteasome subunit alpha type-3	28419.2	5.47E-03	3.89E-02	4.2	
Q6P9V6	Proteasome subunit alpha type-5	26411.0	6.23E-03	4.12E-02	3.4	
P21670	Proteasome subunit alpha type-4	29497.7	6.93E-03	4.40E-02	2.7	
Antioxidant and oxidoreductase activity						
A0A0G2JSH9	Peroxiredoxin-2	21797.7	7.07E-05	6.65E-03	5.2	
P08010	Glutathione S-transferase Mu 2	25702.6	1.22E-03	2.33E-02	3.4	
P07632	Superoxide dismutase [Cu-Zn]	15911.6	2.32E-03	2.85E-02	7.9	
P11232	Thioredoxin	11673.4	2.35E-03	2.85E-02	5.4	
Q9ESH6	Glutaredoxin-1	11878.8	2.60E-03	2.91E-02	9.1	
Q63716	Peroxiredoxin-1	22109.4	2.86E-03	2.99E-02	3.3	
D4A4Y2	Hydroxysteroid (17-beta) dehydrogenase 14	28157.2	1.87E-05	4.97E-03	5.5	
O88989	Malate dehydrogenase, cytoplasmic	36460.0	1.82E-03	2.60E-02	3.3	
P97584	Prostaglandin reductase 1	35695.0	2.62E-03	2.91E-02	3.0	O/OH
P04642	L-lactate dehydrogenase A chain	36450.5	5.47E-03	3.89E-02	2.6	
P04906	Glutathione S-transferase P	23438.8	6.72E-03	4.32E-02	2.8	
Binding activity						
F7F1J0	Odorant-binding protein	10744.3	3.96E-03	2.33E-02	2.9	O/OH/diO/OH
P20289	von Ebner gland protein 1	19725.5	1.09E-03	2.33E-02	4.3	O/OH
Q05982	Nucleoside diphosphate kinase A	17182.0	3.84E-03	3.34E-02	5.3	
D3ZJV7	Lipocalin 4	21646.1	1.15E-02	5.71E-02	2.2	diO/OH
D3ZK46	Lipocalin 3	19557.6	3.94E-03	3.34E-02	2.5	O/OH
D3ZWC2	BPI fold-containing family A, member 5	29169.3	1.65E-03	2.59E-02	5.2	
P02767	Transthyretin	15719.8	1.07E-03	2.33E-02	7.1	
G3V6G1	Immunoglobulin joining chain	17784.3	7.20E-03	4.51E-02	2.5	
B3EY86	Lipocalin 11	20006.5	2.09E-03	2.79E-02	2.9	O/OH
P08699	Galectin-3	27201.5	4.30E-03	3.42E-02	3.6	
F1M6Y6	Lipocln_cytosolic_FA	20372.5	2.80E-03	2.99E-02	3.0	diO/OH
Q9QYU9	Odorant-binding protein	19725.1	3.96E-03	3.34E-02	2.9	O/HO
A0A0G2JZ62	Odorant-binding protein 2B	20507.7	5.39E-03	3.89E-02	2.8	O/OH/diO/OH
P48199	C-reactive protein	25468.0	8.52E-03	4.78E-02	2.9	
P08937	Odorant-binding protein	19699.0	6.26E-03	4.12E-02	2.8	O/OH/ diO/OH
Hydrolase activity						
Q6Q0N1	Cytosolic nonspecific dipeptidase	52693.1	1.31E-03	2.38E-02	6.2	
A0A0G2K4B4	Acid phosphatase, prostate	47941.4	8.79E-03	4.83E-02	4.8	
Q6IN37	GM2 ganglioside activator	21493.0	1.65E-03	2.59E-02	4.0	
Q66HG3	Beta-Ala-His dipeptidase	54927.7	1.34E-03	2.38E-02	5.1	
D3ZZ65	Caspase 14	28255.2	4.10E-03	3.34E-02	2.9	
D3ZSZ1	Poly(U)-specific endoribonuclease	47033.5	3.50E-03	3.34E-02	5.0	
F1M091	Kallikrein	27260.6	7.07E-04	2.10E-02	3.8	
Q6IE55	Glandular kallikrein-10	31221.1	8.51E-03	4.78E-02	2.4	
P00697	Lysozyme C-1	16729.0	7.40E-03	4.52E-02	7.7	

Table 1 (continued) | Salivary proteins overrepresented in 9 rats without cheese LAB persistence at the end of the pre-diet period compared to the 9 rats with LAB persistence

UniProt: accession number	Protein name	Mass (Da)	Anova (p value)	q -value	Fold change	Oxidations
Lyase activity						
P19945	60 S acidic ribosomal protein P0	34215.4	9.84E-04	2.33E-02	4.6	
Q66H59	N-acetylneuraminase lyase	35115.4	1.78E-03	2.60E-02	3.6	
P06214	Delta-aminolevulinic acid dehydratase	36031.5	1.85E-03	2.60E-02	3.3	
Q6P7Q4	Lactoylglutathione lyase	20819.6	1.18E-03	2.33E-02	4.7	
F1MAA9	Serpin family B member 3	35115.4	1.53E-03	2.60E-02	3.6	O/OH
Transferase activity						
P14668	Annexin A5	35744.5	1.04E-04	7.96E-03	5.5	
P13221	Aspartate aminotransferase	46400.0	6.52E-04	2.10E-02	5.9	
P42930	Heat shock protein beta-1	22892.6	7.55E-03	4.52E-02	5.0	
P16638	ATP-citrate synthase	120635.9	6.07E-03	4.12E-02	2.5	
Q9EQS0	Transaldolase	37436.0	8.72E-03	4.83E-02	3.0	
P27605	Hypoxanthine-guanine phosphoribosyltransferase	24477.2	5.80E-05	6.65E-03	4.0	
P85973	Purine nucleoside phosphorylase	32301.9	5.58E-04	2.10E-02	3.1	
P22283	Cystatin-related protein 2	21013.4	5.72E-03	4.01E-02	17.9	
Q497B0	Omega-amidase NIT2	30700.9	7.48E-03	4.52E-02	5.7	
Regulatory and structural activity						
P02401	60 S acidic ribosomal protein P2	11691.9	4.52E-07	2.41E-04	17.1	
D3ZAJ1	Cystatin A (Stefin A)	11034.5	6.41E-03	4.17E-02	5.0	
F1LQM1	Major urinary protein 5	20978.1	4.73E-03	3.65E-02	3.2	
Q5U206	Calmodulin-like protein 3	16802.5	8.40E-03	4.78E-02	9.0	
Isomerase activity						
P25113	Phosphoglycerate mutase 1	28831.9	2.62E-03	2.91E-02	3.8	
D3ZKA0	Serine (Or cysteine) peptidase inhibitor	44249.2	2.48E-03	2.91E-02	4.2	
D4A7L6	Ribose-5-phosphate isomerase	32471.9	3.46E-03	3.34E-02	3.8	
Other functions						
P07151	Beta-2-microglobulin	13719.9	3.95E-03	3.34E-02	2.7	
Q5I0D1	Glyoxalase domain-containing protein 4	33267.6	1.66E-04	1.09E-02	6.1	
A0A096MJ41	FAM3 metabolism-regulating-signalling molecule B	26407.3	4.80E-03	3.66E-02	2.9	
D4AE00	AP-3 complex subunit beta	119142.3	4.04E-03	3.34E-02	3.2	
D4A0S3	Proline-rich 4	26856.1	5.34E-04	2.10E-02	4.2	O/OH
P50116	Protein S100-A9	13144.9	4.00E-03	3.34E-02	3.4	
Q9JJI3	Alpha-2u globulin	20650.7	2.29E-03	2.85E-02	3.2	diO/OH

Proteins were classified by function according to the Panther (www.pantherdb.org/) online program. All of the proteins are eukaryotic from the species *Rattus norvegicus*. All of the proteins are overrepresented in the saliva of animals without LAB persistence. Only oxidations were searched and were abbreviated as follows: O/OH: Oxidation/Hydroxylation or diO/OH: dihydroxylation. Empty cells indicate no oxidations. Differences in the salivary relative protein levels of animals with and without LAB cheese were compared by ANOVA tests (FDR-adjusted p value and q-value). Proteins with a qvalue <0.05 (p value <0.01) were considered significantly different.

determining the persistence of LAB in the oral environment. Whenever LAB can persist in this environment, they could have the potential to synergistically enhance their antioxidant capacities with the host's antioxidant response.

Considering the high potential of fermented foods from a sustainable and healthy point of view, the development of these types of food is highly increasing. Our work points out the high interindividual variability in the hosting of fermented food bacteria in the oral cavity and highlights the role of the sensitivity to oxidative stress on the persistence of LAB in the context of a fermented food-based diet. Therefore, this work opens new perspectives on the necessity to take in account the physiological factors associated with this variability, particularly in the field of personalized nutrition.

Methods

Cheese production and sterilisation process

Pressed-cooked cheese was produced by using *Streptococcus thermophilus* FTS2, *Lactococcus subsp. lactis* SRTA 2006, and *Lactocaseibacillus paracasei* ATCC 334 as starters, and they were partly ionised, as previously described in. Non-ionised and ionised cheese bars (125 g, vacuum packed) were stored at -20°C and unfrozen at room temperature before being given to animals. The viability of cheese bacteria was quantified by bacterial culture as described in Ibarlucea-Jerez, et al.¹⁰. The concentration of culturable LAB after freezing storage was 1.0×10^9 CFU/g for *Lactococcus*, 1.7×10^8 CFU/g for *Streptococcus*, and 1.7×10^8 CFU/g for *Lactobacillus*. Bacterial counts in the ionised cheese reached less than 100 CFU/g for each bacterial strain.

Animal experimental design

Experiments were conducted on ten-week-old male Wistar rats ($n = 81$) (Janvier, Le Genest Saint Isle, France). Animals were housed individually in polycarbonate cages and acclimated for two weeks to the following animal facility conditions: 22 ± 1 °C; 55–60% hygrometry, and reverse light cycle (50 lux, 12 h: 12 h dark/light cycles from 3 am to 3 pm). All of the animals received water ad libitum and an autoclaved regular chow diet (Ssniff Spezialdiäten GmbH, Soest, Germany). The experimental design was in accordance with the relevant guidelines and regulations and obtained the agreement of the local ethics committee of the University of Burgundy (Dijon, France) and the French Ministry of Higher Education and Research (DAP 27646-2020101214594619).

Rats were arbitrarily allocated into one of the three dietary groups ($n = 27/\text{group}$): standard diet group (SD), ionised cheese-diet group (ICD), and cheese-diet group (CD). Each group followed the following three dietary periods: a pre-cheese diet period (2 weeks), a cheese diet period (3 weeks), and a post-cheese diet period (2 weeks). During these time periods, chow diet was given ad libitum to the three groups from 3 pm to 9.30 am (the next day) and was non-accessible for the rest of the day. During the cheese-diet period, the ICD and CD groups received 4 g of ionised and non-ionised cheese, respectively (Fig. 5). Chow was given only after both groups had finished the piece of cheese. Each time period was independently analysed from the others.

Body weight and chow consumption were recorded every 2 days during the three time periods (Supplementary Fig. S1). At the end of each time period, the salivary pH was measured with pH strips (Merck, Darmstadt, Germany) (Supplementary Table S1), and salivary samples were collected at 1 pm from awake animals by using sterile Hydra flock ultrafine swabs (Puritan, Guilford, Maine). Salivary samples⁴⁰ were

collected at least 23 h after the last consumption of cheese at the end of the cheese diet period. Swabs containing saliva were frozen at -80 °C until analyses.

Saliva bacterial profiles

The total volume of saliva collected from the swab generally did not exceed 20 μl . Saliva DNA extraction was performed with the ZymoBIOMICS™ DNA Microprep Kit (Zymo Research, Irvine, California).

qPCR amplifications were conducted in duplicate, utilizing 15 μl reaction mixtures. Each reaction mixture consisted of 3 μl of DNA extract, 800 nM of each primer, and 7.5 μl of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Marnes-la-Coquette, France) within a CFX96 Real-Time PCR Detection System (Bio-Rad, Marnes-la Coquette, France). The thermal cycling conditions included an initial denaturation at 98°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. The primers used for relative gene quantification of the three bacteria species were: *L. lactis tuf* gene (LLTUF forward: TGAAGAATTGATGGAACCTCG / LLTUF reverse CATTGTGGTTCACCGTTC)⁴¹, *L. paracasei leuS* gene (LCLEUS forward: GCTGGATGCTGGTATTGCTT LCLEUS reverse ATCACGCAGTTTGCCCTTCAT)⁴⁰, *S. thermophilus panE* gene (STPANE forward: CCTCGGTGCTCAAGTGGATT STPANE reverse TGGTGTC TGAATGCTAATTGGA)⁴². The quantifications obtained using the cheese LAB-specific primers were compared to the quantification obtained from all bacteria primers targeting the 16S gene (SPU926 forward: AAACCAAAGAATTGACGG, SPU1062 reverse: CTCACRRCACGAG CTGAC)⁴³. The use of these primers, as well as, the following equations allowing to calculate the relative percentage of each LAB bacteria species were described in Ibarlucea-Jerez, et al.¹⁰

Amplification of the V3-V4 hypervariable region of the 16S rRNA gene was performed by using Phanta[®] Max Super-Fidelity DNA Polymerase (Vazyme) and the primers V3PCR1F_460 bp: 5'-ACGGGAGGCAGCAG-3' and V4PCR1R_460 bp: 5'-TACCAGGGTATCTAATCC-3'. The PCR mix contained 50 ng of DNA, 1 μl of dNTPs (10 mM), 1 μl of each primer (20 μM), and 1 μl of Taq polymerase in a total volume of 50 μl . The utilized program used the following parameters: 95 °C for 3 min, followed by 35 cycles at 95 °C for 15 s, 65 °C for 15 s, 72 °C for 60 s, and a final extension at 72 °C for 5 min. As DNA concentrations were low, a second PCR was needed. For this PCR procedure, 5 μl of the previously purified DNA was taken and multiplexed by using the same primers under the same conditions as the abovementioned PCR. Purified amplicons were sequenced by using MiSeq sequencing technology (Illumina, San Diego, CA, USA) on the @BRIDGe platform (INRAE, Jouy-en-Josas, France). The paired-end reads obtained from MiSeq sequencing were treated as described in Lecomte, et al.⁴⁴.

A 16 S rRNA amplicon bioinformatic analysis was performed by using R software v4.2.1 (R Core Team, 2017); specifically, we used Rstudio and the following R packages: ggplot2 v3.3.5⁴⁵ and phyloseq v1.40.0⁴⁶. Differential abundance tests were performed by using DESeq2 v1.32.0⁴⁷.

All of the samples were rarefied to even the sampling depths before computing within-sample compositional diversities (observed richness) and between-sample compositional diversity (Bray–Curtis dissimilarities).

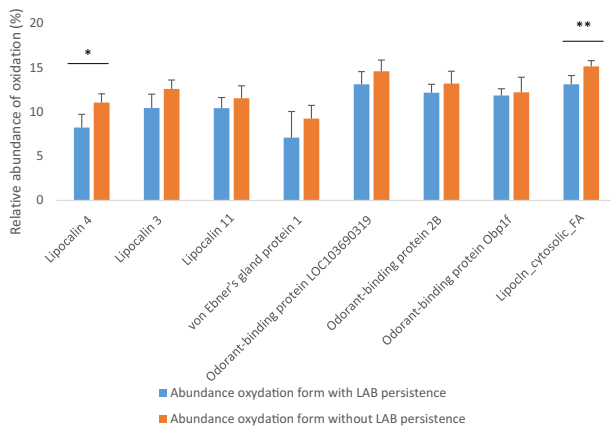
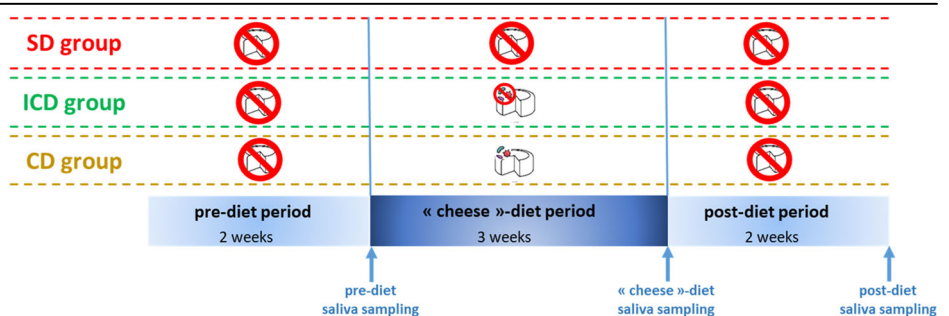


Fig. 4 | Relative abundance of the oxidised form of the proteins with binding activity. The relative abundance (%) is presented as the mean \pm standard deviation ($n = 9/\text{group}$) in the LAB persistence (blue) and no persistence groups (orange). Results were compared by using ANOVA tests. Proteins with a q -value < 0.05 (p -value < 0.01) were significantly different.

Fig. 5 | Feeding and saliva sampling protocol for the three experimental groups. The Standard Diet group (SD), Ionised Cheese-Diet group (ICD), and Cheese-Diet group (CD received chow diet during the three periods ($n = 27/\text{group}$). ICD and CD groups received a portion of 4 g of ionised and non-ionised cheese respectively only during the “cheese”-diet period. Saliva samplings were performed at the end of each period on all animals.



A principal coordinate analysis (PCoA) was performed on Bray–Curtis dissimilarities. Raw, unrarefied OTU counts were used to produce relative abundance graphs. Observed richness data were analysed by using 1-way ANOVA. A permutational multivariate analysis of variance (PCoA) (PERMANOVA) test was performed on the Bray–Curtis matrices by using 9999 random permutations. Phylum, family, and genus relative abundances were compared by using a Kruskal–Wallis test followed by the Dunn’s test. OTUs that presented an adjusted $p < 0.05$ were declared as being significantly abundant.

Saliva proteome analysis

Eighteen saliva samples that were collected in the cheese-diet group during the pre-diet period were prepared for LC-MS/MS analyses. The salivary samples were originated from the saliva of rats presenting cheese bacteria persistence (101, 107, 115, 116, 120, 121, 123, 124, 126) and saliva from rats without bacterial persistence (103, 109, 110, 111, 114, 118, 119, 125, 127). The swabs containing saliva were centrifuged at $14,000 \times g$ for 20 min, with the head swab pointing outwards from the tube. Protein concentration was measured by using a Nanodrop spectrometer (Thermo Scientific, USA). Additionally, samples were diluted in Milli-Q water (Millipore, Burlington, MA, USA) to adjust all of the samples to the same protein concentration, after which they were mixed with 1 volume of Laemmli denaturing buffer and heated at 90°C for 5 min. Samples were loaded onto SDS-PAGE gels containing 12% and 5% acrylamide in resolving and stacking gels, respectively. Electrophoresis was performed by using a Mini-Protean II unit (Bio-Rad, Marnes-La-Coquette, France) at 70 V until the dye front entered the resolving gel. Gels were fixed for 15 min in a solution containing 65% Milli-Q water, 30% ethanol, and 5% acetic acid and stained for 1 h in R-250 Coomassie. After overnight decolouration in Milli-Q water, bands were manually excised. Subsequently, bands were reduced in 10 mM dithiothreitol in 50 mM ammonium bicarbonate and alkylated in 55 mM iodoacetamide in 50 mM ammonium bicarbonate. Destaining of the excised bands was obtained via successive rinses in 25 mM ammonium bicarbonate/acetone nitrile (1:1 v/v). Gel pieces were then dried via incubation in 100% acetone nitrile for 10 min followed by vacuum-drying in a SpeedVac. Finally, gel pieces were incubated overnight at 37°C with $30\ \mu\text{L}$ of a trypsin solution (V5111, Promega) at $10\ \text{ng}/\mu\text{L}$ in 25 mM ammonium bicarbonate. Peptide extraction was performed via the addition of $40\ \mu\text{L}$ of 100% acetone nitrile and 0.5% formic acid, followed by sonication for 15 min. The trypsin digests were vacuum-dried in a SpeedVac.

Each sample was solubilised with $20\ \mu\text{L}$ of a 0.1% TFA solution containing 1.2 pmoles of Promega isotopologue peptides (Peptide Reference Mix, V7491) and transferred into HPLC vials before LC-MS/MS analysis. Five microlitres of each hydrolysate were injected into the nano HPLC; after a wash and concentration step (pepmap, $300\ \mu\text{m}$, $0.5\ \text{mm}$) in 0.05% TFA water, the peptides were separated on a C18 analytical column (Acclaim PepMap, $75\ \mu\text{m}$, $25\ \text{cm}$, ThermoScientific) through a gradient of acetonitrile (ACN/FA, 99.9/0.1) from 2–25% in 60 min. The eluted peptides were nano-electrosprayed into an Orbitrap Q Exactive HFX mass spectrometer (ThermoScientific) operating in data-dependent mode. The parent ion was selected in the Orbitrap at a resolution of 60,000, and an MS analysis was followed by 18 MS/MS with MS/MS fragment analysis at a resolution of 15,000.

The raw data from each analysis were imported into LCProQI label-free quantification software (Progenesis, Waters). After alignment and peak-picking steps that allowed for the comparison of identical ionic signals in each run, the ionic maps were normalised (housekeeping method by using LCProQI) based on the signals of the isotopologue peptides that were present in each sample in the same quantity (300 fmoles). All of the detected ions were subsequently quantified, and the chosen comparison designs allowed for a statistical study of the entire detected ion map. The identification was carried out by MASCOT (2.7, www.matrixscience.com) and PEAKS (XPro, www.bioinform.com) by exporting the MS/MS spectra, $\text{rank} < 20$ that was obtained during the entire LC-MS/MS runs. The interrogation parameters were set as follows: a mass tolerance of 10 ppm for the parent ion and 0.02 Da (PEAKS) or 10 ppm (MASCOT) for the

fragment ions. The possible oxidation of methionines, as well as the possible deamidation of asparagines (N) and/or glutamines (D), were also indicated in the search. Peptides were considered to be identified in a eukaryote protein database (UniProt_2104, *Rattus Norvegicus* [29,934 sequences]) when the PEAKS or MASCOT score that was obtained allowed a false discovery rate of less than 1%.

The identification results were reimported into LCProQI, which allowed for the identification of quantified ions. Only proteins with at least two identified peptides were validated as being present in the samples (Supplementary Table S2). The relative quantification of each identified protein was calculated by summing the abundance of the most intense unique peptides (Hi-N4 [max], –N3, or –N2 [min] peptides depending on the protein) identifying a single protein. For the study of oxidised proteins, only modified peptides (+15.99 or 31.9898 [dihydroxylation]) were used to calculate the abundance of the oxidised form of the protein.

The comparison of protein abundances between animals presenting LAB persistence or not was studied by ANOVA tests (p -value and q -value FDR adjusted) performed in the WFLCProQI. Proteins with q -values < 0.05 ($p < 0.01$) were considered to be significantly different in the design study. Bioinformatics analyses that were performed on the differential proteins were analysed by using Panther (www.pantherdb.org/) and String (<https://string-db.org>).

Data availability

All of the data supporting the mass spectrometry proteomic data were deposited at the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD041306 (Username: reviewer_pxd041306@ebi.ac.uk, Password: 8zIKMfYz), and the 16S rRNA gene sequencing datasets are available in the following databases: <https://doi.org/10.57745/YZCCPK>.

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Author contributions

MIJ, PG, HL, and EN conceived and designed the experiments. MIJ, MM, and CC performed the experiments. MIJ, MM, and CC analysed the data. MIJ, HL, and EN wrote the paper. EN, HL, and PG managed the funding acquisition.

Competing interests

The authors declare no competing interests.

Additional information

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