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Effect of dairy matrices on the survival of *Streptococcus thermophilus*, *Brevibacterium aurantiacum* and *Hafnia alvei* during digestion

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Abstract

This study evaluated the ability of dairy matrices, different in composition (with and without fat) and structure (liquid and gel), to enhance microorganisms survival through digestion. The viability of three dairy microorganisms *Streptococcus thermophilus*, *Brevibacterium aurantiacum* and *Hafnia alvei* was measured during *in vitro* and *in vivo* digestion. *S. thermophilus* was highly sensitive to gastric stress, and was not found in the duodenal compartment. *B. auranticum* was moderately sensitive to gastric stress but resistant to duodenal stress. *H. alvei* was highly resistant to both stresses. LIVE/DEAD confocal microscopy's images, probed the effect of low pH on microorganisms survival. However, *in vivo* analyses (16S rRNA gene metabarcoding) failed to confirm *in vitro* observations since tested microorganisms were not detected. Despite of the different evolutions during digestion on buffer capacity, lipolysis, and rheological characteristics, we did not observe any protective effect of the dairy matrices on microorganisms survival.

Keywords:

Digestion, microorganisms survival; dairy matrix

1. Introduction

Dairy microorganisms have been recently highlighted because of their ability to survive digestive stress and interact with the intestinal microbiota producing some positive health effects (Adouard et al., 2015; Lay et al., 2004).

Some authors affirm that the presence of a dairy matrix could enhance the survival of these microorganisms during digestion (Do Espirito Santo, Perego, Converti, & Oliveira, 2011; Ranadheera, Evans, Adams, & Baines, 2012; Sanders & Marco, 2009). However, literature reports are contradictory. For instance Faye, Tamburello, Vegarud, and Skeie (2012) observed that survival of *L. lactis ssp. cremoris* during *in vitro* experiments was higher in fermented milk than in synthetic medium. In contrast, Sumeri, Adamberg, Uusna, Sarand, and Paalme (2012) observed that the same microorganisms did not survive during *in vitro* experiments when semi-hard cheese was used. Lay et al. (2004) observed that the viability of *S. thermophilus* during *in vivo* experiments was greater when eaten and grown in Camembert cheese than in yogurt.

Thus, the real effect of the dairy matrix on microorganisms survival has not been fully elucidated. The mechanisms that try to explain this protective effect refers to a) the effect of the microorganism's preadaptation to the matrix b) macrostructure effects (buffer capacity and/or effects on gastric emptying rate) and c) microstructure effects (interactions between microorganisms and dairy components and/or microstructure itself).

Preadaptation effect is related to the inherent mechanism of response to stress expressed during food manufacture that produces a cross-adaptation to further digestive stress (Begley, Gahan, & Hill, 2005; Homayoni Rad, Vaghef Mehrabany, Alipoor, & Vaghef Mehrabany, 2016). Uriot et al. (2016) observed that viability of different strains of *S. thermophilus* to *in vitro* experiments was dependent on the activation of urease and small Heat Shock Proteins.

Meanwhile macrostructure effects are related mostly to the buffer capacity of the matrix during stomach phase. In this regard Gardiner, Ross, Collins, Fitzgerald, and Stanton (1998) suggested that the higher viability of *E. faecium* in Cheddar and yogurt during *in vitro* experiments was due to its higher buffer capacity compared to the synthetic medium.

On the other hand microstructure role was suggested by Sumeri et al. (2012) who observed that survival of lactic acid bacteria during *in vitro* experiments was higher in semi-hard cheeses than in synthetic medium because of the higher protein content and presence of fat globules in dairy that provide a physical barrier, against digestive stress. Our study was developed in this context with the objective to test the ability, beyond preadaptation effect, of dairy matrices (milk and gel with or without fat) to enhance the survival of microorganisms, from dairy origin, *S. thermophilus*, *H. alvei* and *B. auranticum*, during digestion. The *in vitro* digestive model (DIDGI®) was set using the same digestion parameters for all matrices; and the degradation of the matrix components was characterized during this digestion. *In vivo* experiments were also performed to observe the effect of dairy matrices on microorganism survival during full digestive transit.

2. Material and Methods

2.1. Bacterial Strains

Streptococcus thermophilus TIL 257 (*ST*), *Brevibacterium aurantiacum* ATCC9174 (*BA*) and *Hafnia alvei* GB01 (*HA*) were selected by its role during manufacture of cheese. They were obtained from the culture collection of INRA, UMR 782 Genie & Microbiologie des Procédés Alimentaires (F-78850 Thiverval-Grignon, France).

Working cultures were reactivated from frozen stocks stored at -80 °C by transfer *BA* and *HA* to Brain Heart Infusion broth (BHI), and incubation at 25 °C with agitation. *ST* was cultivated anaerobically in M17 at 37 °C. After 48 hours, 50 mL of each culture was mixed with 50 mL of new medium and incubated 24 h for *BA* and *HA* and 18 h for *ST*, until reaching the stationary phase, to a final concentration of 10⁷ cells of each strain introduced into the DIDGI® system.

Viability during digestion was determined after dilution in Maximum Recovery Diluent (MRD, containing NaCl 8.5 g/L and peptones 1g/L) by plating on the same agar based medium used for liquid culture. The same incubation temperatures were used. All growth media were from Biokar Diagnosis (Beauvais, France), MRD was from Difco (Pessac, France).

2.2. Dairy matrices

Four dairy matrices with different composition and structures, skimmed milk (SM), whole milk (WM), rennet gel from skimmed milk (GSM) and rennet gel from whole milk (GWM), were used to compare its protective effect on the survival of microorganisms during dynamic *in vitro* digestion. Synthetic medium (S) prepared with MRD was used as control.

All dairy matrices were prepared with 10.9 g of skimmed milk powder (36.2% protein, 1.5% fat, 56% lactose, 8.5% minerals; Low heat powder, Lactalis, France) diluted in 110.1 mL of sterile CaCl_2 (0.01 M) and stirred for 30 min.

WM was prepared adding 4.4 g of anhydrous milk fat (Lactalis, France), previously melted, to 121 mL of SM and mixed 5 min at 3000 rpm and 50 °C using a Thermomix (TM31, Vorwerk, Nantes, France). Then, the WM (3.8% fat) was cooled rapidly and kept at 4 °C until analysis. GSM and GWM were prepared by mixing 121 mL of SM or WM with 2.4 mL of rennet (1:50, Naturen 450, 555 mg/L of active chymosine, 145 IMCU/mL; Chr. Hansen, Arpajon, France), the mix was gently stirred for a few seconds before being placed at 30 °C for 40 min. Gels rheological characteristics were determined with a rotational rheometer (MCR301, Anton Paar, Uls, France), operating in the oscillatory mode (Kristo et al., 2003, Mat et al., 2015). A mix of 18 mL of milk (SM or WM) and 1.8 mL of microorganisms were introduced in the coaxial cylinder (CC27/T200/SS, Anton Paar, Uls, France) and warmed at 30 °C. Then 400 μL of rennet were added and the evolution of the storage modulus G' and the loss modulus G'' recorded for 60 minutes. The deformation and frequency used were 1% and 1 Hz respectively. After 40 min of rennet addition, G' and G'' were stable and raised 28.7 ± 1.25 Pa and 7.2 ± 0.13 Pa respectively. For the digestion's experiments, 4 mL of each microorganism were included in the matrix immediately before the *in vitro* digestion for liquid matrix (S, SM and WM) and just before the addition of rennet for gel matrices (GSM, GWM). Before the introduction of the matrix in the DIDGI[®], artificial saliva (250 mg of mucine and 100 mg α -amylase in 100 mL of MRD) was added to the matrix to complete 200 mL.

2.3. The *in vitro* dynamic model of the gastrointestinal tract

The Digesteur Dynamique Gastro-Intestinal (DIDGI[®], INRA, UMR 782 GMIPA) was developed to simulate digestion (Ménard et al., 2014) and it consisted of three separated

compartments simulating the stomach, the duodenum and the small intestine controlled by the software STORM for STomach Regulation and Monitoring (Adouard et al., 2016; Guillemin, Perret, Picque, Menard, & Cattenoz, 2010). The digestion parameters used were previously described by Adouard et al. (2016) with some modifications (Table 1). All digestive juice components were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France) and diluted in MRD.

Digestion experiments for each food matrix were performed at least by triplicate. Samples from each compartment were collected at different stages of the digestion. Microbiological counts were made immediately and samples were kept at -20°C to be further analyzed.

2.4. *In vivo digestion*

Twenty four male mice (CH3/HeN) five weeks old were placed in a controlled room (21°C) with a 12 h light/dark cycle. Mice had free access to standard food (SAFE RO3, Augy, France, www.safe-diets.com) and sterilized water. Mice were separated in 3 groups feed differently during the five first days of the experiment. The controlled group (number 116) had only the standard feeding. The matrix group (number 152) and the matrix + microorganisms group (number 131) were respectively intragastric inoculated in the morning with 200 μL of WM and WM + microorganisms. This inoculating period was followed by a seven-day standard feeding period. The feces were collected at days 1, 5 and 12, in the morning, just before inoculation. Samples were stored at -80°C until analysis. All animal experiments were carried out in accordance with the directive 2010/63/EU of the European parliament and the council on the protection of animals used for scientific purposes.

2.5. *Characterization of microorganisms and matrices*

2.5.1. *Metagenomic and metatranscriptomic analysis*

For *in vivo* experiments, DNA extraction was performed from 30 mg of feces as previously described (Godon, Zumstein, Dabert, Habouzit, & Moletta, 1997). The V3-V4 region of the 16S rRNA gene was amplified from 10 ng of purified genomic DNA using the primers F343-TACGGRAGGCAGCAG and R784-TACCAGGGTATCTAATCCT as previously described (Lazuka et al., 2015). Then,

library preparation and sequencing were performed at the GeTPlaGe platform (Toulouse, France) using the Illumina MiSeq technology. Paired-end reads were merged using Flash (Magoč & Salzberg, 2011) and FASTQ files were then quality-filtered and analyzed using QIIME v.1.9.0 (Caporaso et al., 2010). Chimeric sequences were identified using USEARCH and operational taxonomic units (OTUs) were clustered at 97% identity using UCLUST (Edgar, 2010). Any OTU comprising less than 200 reads were removed, as recommended by Bokulich et al. (2013). Representative sequences for each OTU were classified taxonomically using RDP Classifier (Wang, Garrity, Tiedje, & Cole, 2007) against the Greengenes database (McDonald et al., 2012).

2.5.2. Confocal laser scanning microscopy (CLSM).

To observe the microstructure of the matrix (WM and GWM) and the location of microorganisms, milk fat was stained (1 mL of sample) with 100 μ L of non-polar Bodipy® 493/503 lipid probes (working solution concentration at 0.5% w/v in DMSO). Proteins were stained with 100 μ L of amine reactive dye DyLight™ 550 NHS Ester 562/576 (0.5% w/v in DMSO). Microorganisms (1 mL of culture) were stained with 1 μ L of the fluorescent SYTO® 61 nucleic acid stain 628/645 (commercial solution at 5 mM in DMSO). Then 33 μ L of each culture was added to the milk matrix. For gel matrix, 20 μ L of rennet were added to 1 mL of milk and placed at 37 °C for 40 min. After gelation, samples were transferred into 96 wells μ Clear® cell culture microplate (Greiner Bio-One Kremsmünster, Austria).

In a second time, the viability of microorganism during acid stress was determined with a Filmtracer™ LIVE/DEAD® Biofilm Viability Kit including two fluorescent markers, SYTO® 9 and Propidium iodide nucleic acid stains (Hannon, Lopez, Madec, & Lortal, 2006; Stocks, 2004).

The LIVE/DEAD staining was realized only on the synthetic medium, 0.5 μ L of each stain was directly added to 250 μ L of sample in the microplate. Then HCl 0.05M was added to reach the concentration of 2.3 mM, corresponding to pH2 in the synthetic medium.

Samples were incubated in the dark at room temperature for at least 10 minutes for staining, and then mounted on the motorized stage of an inverted confocal microscope (Leica TCS SP8 AOBS, Leica Microsystems) at the MIMA2 platform

(<http://www.jouy.inra.fr/mima2/>). A water immersion objective lens of 63X/1.2 N.A., (working distance 300 µm) was used. 2D sections were acquired at 600 Hz scan speed with bidirectional scan X direction mode, with numerical zoom 1, with 512 x 512 xy image definition (pixel size 361 nm), with a 8-bit dynamic range per pixel, with pinhole at Airy 1 (111.5 µm diameter) and with a z-step of 1 µm.

Image analysis was carried out using ImageJ software V1.49 (NIH, Bethesda, USA).

The microscopic experiments were performed independently from DIDGI®'s one.

2.5.3. Matrix buffer capacity

The amount of HCl used to reach pH2 in the stomach phase of DIDGI® system was determined through the STORM software for each tested matrix. The resulting pH-titration curves were used to calculate the buffer capacity through equation (Eq.1) (Salaün, Mietton, & Gaucheron, 2005).

$$\frac{dB}{dpH} = \frac{\text{Volume of acid (mL)} \times \text{Normality of the acid}}{\text{Changes in pH} \times \text{volume of sample}} \dots \dots \dots \text{Eq. 1}$$

As described by Upreti, Bühlmann, and Metzger (2006), sample's dilution with the acid was considered insignificant for buffering curves.

2.5.4. On line torque measurement

Rheological characteristic of dairy matrices was measured on-line during gastric digestion. These experiments were conducted simultaneously with DIDGI®'s ones with a specific system developed and adapted to the rheometer used before (section 2.2). The system was composed by a glass vessel (high 165 mm, diameter 80 mm) surrounded with a heating mat to control temperature. The stirrer consisted of four stainless steel agitation blades (high 20 mm, long 35 mm) connected to the rheometer. The turbine blades were fully immerse in 200 mL of the matrix in the glass vessel. A pH, redox and temperature probe (Electrode InPro 480i/SG/120, reference 52003581, Mettler Toledo, France) was connected to the STORM software to measure and control the conditions during the experiment.

As described for DIDGI®'s experiments, 200 mL of S, SM, WM, GSM or GWM were introduced in the glass vessel of the rheometer. Rennet gels were cut in cubic pieces

with edges of 1.5 cm just before the introduction. The stirrer and the pH probe were placed in the matrix and the measurement of the torque began. Pepsin and HCl were incorporated in the same conditions than for the digestion (Table 1). The rotating speed of the stirrer was 60 rpm.

2.5.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Prior to analysis, samples kept at -20°C were defrosted and homogenized during 15 s at 15500 rpm using an Ultraturrax (T25, Janke & Kunkel, Staufen, Germany). Samples were stabilized at 25°C for 20 min before analysis.

For the SDS-PAGE analysis, protein contents were calculated using the method reported by Lüthi-Peng and Puhon (1999) with slight modifications. Briefly, 100 μL of sample were diluted in 1900 μL of 6 M guanidine-HCl in 0.1 M Na acetate buffer, pH5.0. The absorbance was measured at 280-320 nm (spectrophotometer Evolution 201, ThermoFisher Scientific, France) and used to calculate the protein content. The calibration curve was performed with the same dairy proteins from the skimmed milk powder.

SDS-PAGE was performed using the 4-12% polyacrylamide NuPAGE SDS-PAGE precast gels (ThermoFisher Scientific, France). Samples were diluted (with running buffer) to reach a final concentration of 10 μg of proteins to be loaded in each lane. Gels were fixed in 700 mL of 20X NuPAGE MES SDS running buffer. Running was at 60 mA/gel and 120 V for 2 h. After washing 3 x 5 min with deionized water, gels were stained 1 h with Colloidal Blue Staining Kit (Simply Blue Safe Stain, ThermoFisher Scientific, France). A low molecular weight marker (SeeBluePlus 2, ThermoFisher Scientific, France) was used to identify the proteins in the gel. At least 2 gels were run of each food matrix. Image analysis of SDS-PAGE gels was carried out using PDQuest 2-D analysis software, version 6.2.1.

2.5.6 Free fatty acid by GC

For free fatty acid (FFA) extraction, 50 μL of sample were mixed with 100 μL of internal standard solution (0.49 μg tridecanoic acid/mL in CHCl_3) and dehydrated with Na_2SO_4 anhydrous; then extracted twice with 1 mL of diethyl ether/heptane (1:1 v/v)

and shaken vigorously for 3 min. The organic supernatant was separated and both extracts were combined.

Solid phase extraction (SPE) of FFA was performed as described by Voigt, Chevalier, Qian, and Kelly (2010). Solvent was evaporated to dryness with a speed-vac system (ThermoFisher Scientific, France) and dissolved with 100 μ L of CHCl_3 , put in a tight closed micro-vials.

FFA extracts were analyzed by gas chromatography (Agilent Technologies 6890, Palo Alto, Ca.) coupled with a mass selective detector (MS 5973N, Agilent Technologies, France) and a CTC autosampler (CombiPAL, Agilent Technologies, France).

FFA were separated with a DB-FFAP column (30 m x 250 μ m x 0.25 μ m, Agilent Technologies, France) using Helium as carrier gas at 1.2 mL/min. Oven temperature was set at 40 $^{\circ}\text{C}$ for 1 min, then increased at 10 $^{\circ}\text{C}/\text{min}$ to 240 $^{\circ}\text{C}$ and hold 30 min. Injector was set at 250 $^{\circ}\text{C}$ in splitless mode and 1 μ L sample was injected. Mass spectra were recorded at 70 mV in the total ion mode (SCAN) with an m/z 33-550.

Quantification was performed with the internal standard method. FFA reference compounds were provided by Sigma-Aldrich with a minimum purity of 98%. Standard calibration curves had a concentration of 0.35-935.0 $\mu\text{g}/\text{mL}$ and were considered satisfactory when $r^2 \geq 0.980$.

2.6. Statistics analysis

Statistical analysis was performed using Statistica V12 (Statsoft Inc., Tulsa, OK, USA). Results were expressed as the mean \pm standard deviation of the three biological independent experiments and were compared using a Student's test. Differences were considered statistically significant when $p < 0.01$. The comparison of the strains survival rates was performed independently in each compartment. For each strain, at each sampling time, Student's test was used to study the effect of the medium. For each strain in one medium, Student's test was used to study the effect of the time. For FFA, analysis of variance (ANOVA) and the Fisher's least square differences (LSD) were applied, they were considered statistically different when $p < 0.05$.

3. Results

3.1. Survival of the microorganism to digestion

3.1.1. *In vitro* experiments.

The effect of *in vitro* digestive stress on the survival of *HA*, *BA* and *ST* was tested in the dynamic digester DIDGI® and the protective effects of different dairy matrices (S, SM, WM, GSM, GWM) were compared (Figure 2 and 3). The strains exhibited different behavior in each digestive compartment.

3.1.1.1. *Hafnia alvei*

HA was the most resistant microorganism during all the digestion process. In stomach it remained constant during the first 60 min (Figure 1A) whatever the matrices with no significant statistical difference except at 60 min for GSM. Then, at 70 min, the viability began to decrease with an average loss of 2.6 log CFU/mL at the end of gastric phase. Slight differences between matrices were observed at the end of this phase (100 min). The viability decreased in all the matrices to less than 5 log CFU/mL except in SM media, for which the level was 5.56 log CFU/mL. The strong resistance of *HA* to acid stress was confirmed by LIVE/DEAD results with only 2% of microorganism with compromised membranes (red color) after 20 min incubation at pH2 (Figure 3A).

During the duodenal phase, *HA* showed also strong resistance and the viability remained constant between 6.5 to 7.2 log CFU/mL for the first 60 min (Figure 1B). Then, it fell losing on average 1.03 log CFU/mL at the end of this phase. Slight differences were observed between matrices at 120 min. S and WM media exhibited higher viability (6.37 log CFU/mL) than other matrices (5.57 log CFU/mL), however these differences were not significant.

HA showed strong resistance to the stress in the intestinal phase (Figure 1C). All the matrices displayed similar initial level of viability in the small intestine (7.06 ± 0.05 log CFU/mL at 50 min.). Levels in S, WM, GSM and GWM remained constant over 140 min of intestinal digestion; while in SM loss of viability was more evident. At 170 min, there were significant differences ($p < 0.01$) between matrices. S, SM and GSM viability remained statistically constant. The counts of *HA* in WM fell gradually after 170 min until lose 2.6 log CFU/mL by the end of the experiment. The same behavior was observed in GWM. After 200 min, the level of *HA* had a significant drop of viability (1.3 log CFU/mL) compared with the initial value at 50 min (Figure 1C).

Viability of *HA* could be attributed to the presences of an outer membrane, characteristic of all the gram-negative bacteria that provide them with an extra protection to all kind of environmental stresses. Additionally *HA* is an ornithine and urease positive bacteria (Janda & Abbott, 2006), both enzyme increases the pH through decarboxylation and production of ammoniac respectively. However both enzymes could be later inactivated by the action of the enzymes present in digestion, thus the real mechanism of response of *HA* could be more related to genetic adaptation. By its approach with *E. coli* we could attribute its high survival rate to the effect of efflux pumps or a cross-adaptation to the previous mechanism related to the acidity stress (Begley et al., 2005).

3.1.1.2. *Brevibacterium aurantiacum*

BA was sensitive to gastric stress as shown in figure 2A. For each medium, its viability decreased slightly, 0.38 log CFU/mL during the first 30 min. Slight statistical differences between matrices were observed during the first 30 min of gastric digestion. When pH reached to 2.7 (50 min), viability decreases on average 1.25 log CFU/mL without any statistically significant difference ($p < 0.01$) between matrices. At 60 min *BA* was only found in SM at 3.92 ± 0.97 log CFU/mL. However, high numbers of microorganisms were capable to reach the duodenal phase during the first thirty minutes (7.9 and 8.6 log CFU). Moderately sensitivity of *BA* to acid stress was also observed during LIVE/DEAD experiments with 11% of damaged cells (Figure 3B). Gastric sensitivity of this microorganism was not surprising since during cheese ripening it grows only when cheese pH reaches 6–7 (Irlinger et al., 2012).

BA was resistant to duodenal stress (Figure 2B), its viability decreases constantly, losing only on average 1.73 log CFU/mL in all matrices over the duodenal exposure. The drop was faster for SM and GWM matrices which exhibited the lowest levels at 120 min.

When *BA* reached the intestine, its initial concentration was between 6.71 log CFU/mL and 5.47 log CFU/mL respectively for WM and SM matrix (Figure 2C). All the matrices displayed similar behavior until 140 min. Differences observed between the matrices during this time were not statistically significant and attributed to differences in the initial count in this compartment. After 140 min, significant differences between

matrices were observed. From 170 min to 230 min, *BA* could only been found in S and SM, meanwhile the viability of WM, GSM and GWM descended below the detection limit of the plating method.

This behavior can be related with *BA* ability to survive in cheese surface even when it is salted in many occasions (Irlinger et al., 2012). Genoma of *B. auranticum* 9174 exhibits an important number of osmoprotectant transporters (Monnet, Landaud, Bonnarme, & Swennen, 2014) which might provide it a further cross-adaptation to other stress (Begley et al., 2005).

3.1.1.3. *Streptococcus thermophilus*

ST was highly sensitive to the gastric phase remaining viable only 30 min in the stomach (4.39 ± 1.7 log CFU/mL). Within the next 10 minutes (below pH4.6) the concentration decreased quickly dropping under our detection limit (data not shown). LIVE/DEAD staining confirmed the sensitivity of *ST* with 40% of damaged cells after 20 min at pH2 (Figure 3C).

These results are in accordance with Adouard et al. (2016) who observed that *ST* did not survive to neither gastric *in vitro* digestion and partially in agreement with those of Marteau, Minekus, Havenaar, and Huis In't Veld (1997) and García-Hernández, Moreno, Chuan, and Hernández (2012) who observed that *ST* survived only briefly in the gastric phase during *in vitro* digestion of yogurt.

In the subsequent compartments of the DIDGI[®], *ST* was no longer found in any of the tested matrices.

3.1.2. *In vivo* experiments.

A total of 3,103,358 16S rRNA (V3-V4 regions) sequencing reads were generated from the 72 mice fecal samples. An average of 36,577 sequences per sample were obtained after quality filtering and chimera removal. They were clustered into 244 bacterial OTUs with an abundance >200 sequences in the entire dataset.

Our *in vivo* analysis failed to demonstrate the survival of *BA*, *HA* and *ST* after digestion. Traces of *Streptococcus* were found in feces but were further attributed to a similar strain part of the GI tract (*S. salivarius* subsp. *salivarius*) as previously suggested by Bogovic-Matijasic et al. (2015). Our results are in agreement with those of del Campo

et al. (2005) who observed that *ST* part of fresh yogurt, was not capable to survive through *in vivo* digestion; and in disagreement with those of Lay et al. (2004) who found that when part of Camembert cheese *ST* was capable to survive *in vivo* digestion but not when it was part of fermented milk; suggesting that a solid matrix was more effective than a liquid one. However in our results, we did not observe any increase in viability when gel was used instead of milk.

The bacterial community structure did not vary neither according to the day of sampling nor the food matrix used for feeding (Figure 4), which indicated that the treatment did not modify the overall composition of the mouse microbiota.

3.2. Chemical and physical characterization of matrices during the digestion

The initials states of WM and GWM structure were visualized by CLSM just after mixing with the strains (pHi= 6.5). Milk proteins were colored in yellow and had a characteristic structure of milk protein powder particles as describe by Mimouni, Deeth, Whittaker, Gidley, and Bhandari (2010). Fat spherical globules appeared in green and microorganisms in bright red color (Figure 3D). When the milk was coagulated by rennet, proteins formed a porous network surrounded by lactoserum (unstained components) and containing different sizes fat globules (Figure 3E). The distribution of microorganisms was not different between liquid and gel matrices.

3.2.1. Protein profiles during *in vitro* digestion

SDS-PAGE profiles are shown in figure 5. Dairy matrices with or without fat presented very similar trends therefore only results with fat are shown. Caseins were hydrolyzed all along the stomach phase without significant differences between gel and milk matrices. At 10 minutes, 10 to 15% of the caseins were hydrolyzed and at the end of the stomach phase 70% remained intact (figure 6A).

This is in accordance with the result of Inglingstad et al. (2010) and Rinaldi, Gauthier, Britten, and Turgeon (2014) while working with *in vitro* digestion of proteins from different animal origin in liquid and semi-liquid matrixes, respectively. In contrast, our results are much higher than those reported by Gallier, Ye, and Singh (2012) during the *in vitro* digestion of bovine milk and Bourlieu et al. (2015) during the *in vivo* digestion of a standardized milk emulsion. These differences can probably be explained by the

milk origin and preparation; as well as the type, amount and activity of the enzymes used in the experiments (Almaas et al., 2006).

In our work, the whey proteins remained intact during the gastric digestion (figure 5 and 6B), as observed by Kopf-Bolan et al. (2014) when they studied the impact of milk processing on the generation of peptides during digestion.

3.2.2. Free fatty acid profiles

Free fatty acid (FFA) released during digestion of WM and GWM are shown in figure 8. For both matrices the most abundant FFA were C16:0, C18:1 n9, and C18. Short chain FFA (C6-C10) showed low concentrations throughout digestion.

For both WM and GWM, all the analyzed FFA showed significant differences ($p < 0.05$) according with the digestion time except C18:2 in WM. Instead, significant differences were observed between matrices for all the FFA except for C6 and C20:0. Globally, FFA release was higher in WM than in GWM except at the end of digestion (230 min) where values were higher in GWM. Both matrices showed a lower FFA release during the stomach digestion than in the intestinal phase.

In the stomach, lipolysis rate was significantly higher for WM than GWM for most of the analyzed FFA. For WM, FFA concentration remained steady throughout gastric digestion, while for GWM it was higher at the beginning of digestion remaining steady between 30 and 60 min. The lower release of FFA observed during the stomach lipolysis could be attributed to the *Rhizopus oryzae* lipase used and to the lack of pregastric lipase in the artificial saliva. Nevertheless, we observed higher values in stomach lipolysis for both WM and WMG than those reported by Devle et al. (2014) for milk digestion using human gastrointestinal juice.

Meanwhile, in the intestine the lipolysis was higher than in the stomach for both matrices. For WM (Figure 7B) the behavior depended on the chain length, short chain FFA (C6-C12) remained steady between 50 and 110 min increasing at the end of intestinal phase; medium chain saturated FFA (C14-C18) and C16:1 fluctuated throughout intestine digestion; while C20 increased constantly. For GWM (Figure 7A), most of the evaluated FFA increased steadily throughout digestion reaching higher values than in WM except for C16:1 and C20 that remained constant and increased only

at the end of intestine digestion. The release and profiles of FFA in the intestine for both WM and WMG were similar to the results reported before by DePeters, German, Taylor, Essex, and Perez-Monti (2001) and Devle et al. (2014) for milk digestion with human duodenal juices. Even though the antimicrobial activity attributed to C6 and C8 against gram-negative bacteria (Turgeon & Rioux, 2011), no significant correlations were found between the individual FFA and *HA* survival at the intestine. The concentrations obtained in this study were inferior to the minimum inhibitory concentration reported by Sun, O'Connor, and Robertson (2002).

3.2.3. Rheological properties of milks and gels during digestion

The evolution of torque of all matrices was measured during gastric digestion (Figure 8). To highlight the role of the digestion on the evolution of the matrix, effect of cutting and stirring on the rennet gel was tested replacing pepsin and HCl by water (Gel-Mechanical action only in figure 8). In these conditions without enzyme and acidification, initial torque of gel was 0.6 mN/m, descended slowly to 0.4 mN/m at 60 min and remained steady until the end. Cutting gel creates more surface where syneresis can occur (Janhoj & Qvist, 2010) and stirring enhance syneresis by preventing sedimentation and rising pressure on the curd (Guinee & O'Callaghan, 2010).

Since there were no differences between matrices with or without fat, the average and standard error of the torque for milk and rennet gel with and without fat were calculated. Contrary to the observations of Devle et al. (2012) who founded differences in viscosity between skim milk and homogenized full fat milk. The authors attributed the differences observed to an effect of softening by the large fat globule. Besides, we can think that our module of torque measure is not enough sensitive to differentiate low gap of viscosity.

Significant differences were observed between milk and rennet gel. For milk matrix, torque decreased from 0.15 to 0.05 mN/m after 40 min and remained steady. This behavior might be due to the effect of stirring, that did not allow the formation of a homogeneous acid curd. However we observed flocculation in the reactor.

For rennet gel the torque decreased swiftly from the initial 0.6 to 0.5 mN/m in the first minutes of digestion. Then it continued decreasing linearly during 50 min until 0.15 nM/m. Between 55–70 minutes the signal increased slightly to 0.18 mN/m. After this period, torque went back down slowly and remained steady until the end of the experiment. As described by Johnson and Law (2010), the HCl addition conduct to low pH in the vessel, reducing the water present in the casein micelles and solubilizing calcium. Thus, the structure of the micelles changed, forming new bonds between caseins, reducing the size of the curd and increasing its permeability; therefore syneresis increased (Barbé et al., 2014). This phenomenon was relatively slow until pH5.5, but when pH decreased, syneresis increased and reached its maximum at pH4.7-4.9 probably due to a reduction of overall charge as the isoelectric point of the casein is approached.

The decrease of torque below pH4.7 also might be attributed to the action of pepsin, since the optimum pH zone is 2-4 (Schlamowitz & Peterson, 1959). The key role of the pepsin on the reduction of viscosity has been previously described by Prakash, Ma, and Bhandari (2014).

The further increase of torque between 60-80 min might be attributed to the aggregation of the micelles. During this phase, the kinetics of aggregation became more important than the hydrolysis by the pepsin and the size of the aggregates increased as the viscosity, as observed for the milk but with a lesser intensity.

3.2.4. Matrices buffering capacity

For *in vitro* digestions, the quantity of acid needed to reach pH2 in the stomach phase was dependent on the type of matrix. Thus, the quantity of HCl used increased proportionally to the complexity of the matrix, imitating the *in vivo* conditions where the acid secretion in the stomach is highly related to the nature of the bolus (Kong & Singh, 2008). However, fat addition in our dairy matrices was not significant for acidification in the gastric phase. The average amount of HCl necessary to reach pH2 for milks (SM, WM) and for gels (GSM, GWM) was respectively 10.6 ± 0.02 mM and 11.5 ± 0.6 mM. For the synthetic medium only 2.7 ± 0.4 mM of HCl were sufficient to reach pH2.

The buffer capacity (dB/dpH) in the gastric phase was determined mathematically from the pH titration curves measured in the DIDGI[®] system (Figure 9).

When the synthetic medium, containing only low concentrations of NaCl and peptones, was acidified from pH6.5 to 2, dB/dpH remained stable from pH5.5 to 3.5 (dB/dpH < 0.005). Then, it increased to reach 0.0125 at pH2.2.

The buffering capacity of milk increased from 0.08 at pH6.5 to 0.017 at pH5.2, its maximum value. Then, dB/dpH decreased to 0.015 at pH5 and remained stable until the end of the experiment. For gels, the buffering capacity rise from about 0.09 at pH6.5 to 0.024 at pH2.75 and then decreased to 0.015 at pH2.

Between pH6.5 and pH5, dB/dpH was higher for milks than for gels. For pH lower than 5, we observed the opposite phenomenon.

Our dB/dpH curves were similar to those reported by Salaün et al. (2005) and Upreti et al. (2006) who found two maximal dB/dpH. Lucey, Hauth, Gorry, and Fox (1993) attributed the peak at about pH5–5.5 to the solubilization of colloidal calcium phosphate and a strong increase below pH3 due to acidic amino acids present in caseins. In rennet-curdled skim milk, Whittier (1929) described a maximal dB/dpH at pH4.7 and the difference with milk was ascribed to the production of para casein by the rennet and to the coagulum structure. Lucey et al. (1993) found a buffering peak at pH4.2 during the titration of rennet whey.

Furthermore it is well known that buffer capacity depends of the type of dairy product (Salaün et al., 2005; Upreti et al., 2006) since matrix with the same components have not necessarily the same structural organization or same digestion kinetic (Rinaldi et al., 2014; Turgeon & Rioux, 2011). Higher buffer capacity in gel than in liquid could be due to a structural effect, where the curd traps the elements that give the buffer capacity, longer than a liquid matrix (Salaün et al., 2005).

In this regard, two main factors need to be considered, the action of the pepsin during the gastric phase and the dynamic evolution of digestion. Hydrolysis of milk proteins and the peptide released during the *in vitro* gastric digestion can modify the buffer capacity; through increasing the number of available ionizable groups (i.e. acidic amino acids) that before were inaccessible (Salaün et al., 2005). The dynamic functioning of

the *in vitro* digester with a continuous transfer between the various compartments also modifies the composition of the matrix and consequently the buffer capacity.

Still, despite the higher buffer effect of dairy matrices, compared with synthetic medium, in our results we did not observe a protective effect on microorganisms survival through digestion. Our results are in disagreement with the previous works of Conway, Gorbach, and Goldin (1987) and Charteris, Kelly, Morelli, and Collins (1998) who in different works with probiotics suggested that milk proteins improved the gastric transit tolerance of microorganisms, because of its tampon effect. Though, our results are in accordance with the reported by Adouard et al. (2016) who observed that survival of *Corynebacterium casei* and *Staphylococcus equorum* during *in vitro* digestion was similar when contained in cheese or synthetic medium.

4. Conclusions

In this work, we studied the effect of dairy matrices different in composition (with and without fat) and structure (liquid and gel) on microorganisms survival through digestion. The tested microorganisms had different survival rates during digestion.

Therefore our results suggest that protective effect of the dairy matrices during digestion is not dependent of buffer capacity or interactions between microorganisms and dairy matrices (components and microstructure). Suggesting that factors that provide protection during digestion could be strain-dependent. During *in vitro* assays digestion parameters, such as gastric emptying and duration of digestion, were set the same for all the tested matrices; however during *in vivo* digestion, these parameters change according to the type of food matrix.

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Figure captions

Figure 1: Survival of HA in five matrices (S, WM, SM, GWM and GSM) during *in vitro* A) gastric, B) duodenal and C) intestinal compartments. * result of statistical analysis of time effect by matrix, ** result of statistical analysis of media effect at the same time of digestion.

Figure 2: Survival of BA in five matrices (S, WM, SM, GWM and GSM) during *in vitro* A) gastric, B) duodenal and C) intestinal compartments. * result of statistical analysis of time effect by matrix, ** result of statistical analysis of media effect at the same time of digestion.

Figure 3: Confocal Laser Scanning Microscopy images showing the protein (yellow), the fat (green) and the microorganisms (red) in A) WM and in B) GWM. Live (green) and dead (red) cells of C) HA, D) BA and E) ST at pH7 and pH2.

Figure 4: Composition of the bacterial communities from fecal samples of individual mouse according to the day of sampling (d1, d5, d12) and the tested group (116: standard feeding; 131: whole milk, WM; 152: whole milk + microorganisms).

Figure 5: SDS page analysis of the evolution of proteolysis during gastric digestion of A) whole milk and B) rennet gel of whole milk. C: Kda protein standard

Figure 6: Kinetics of proteolysis of A) casein, B) β -lactoglobulin and α -lactalbumin during gastric digestion of milk and gel.

Figure 7: Free Fatty Acids released during digestion of A) GWM and B) WM.

Figure 8: Evolution of the torque during digestion for milk and rennet gel matrices.

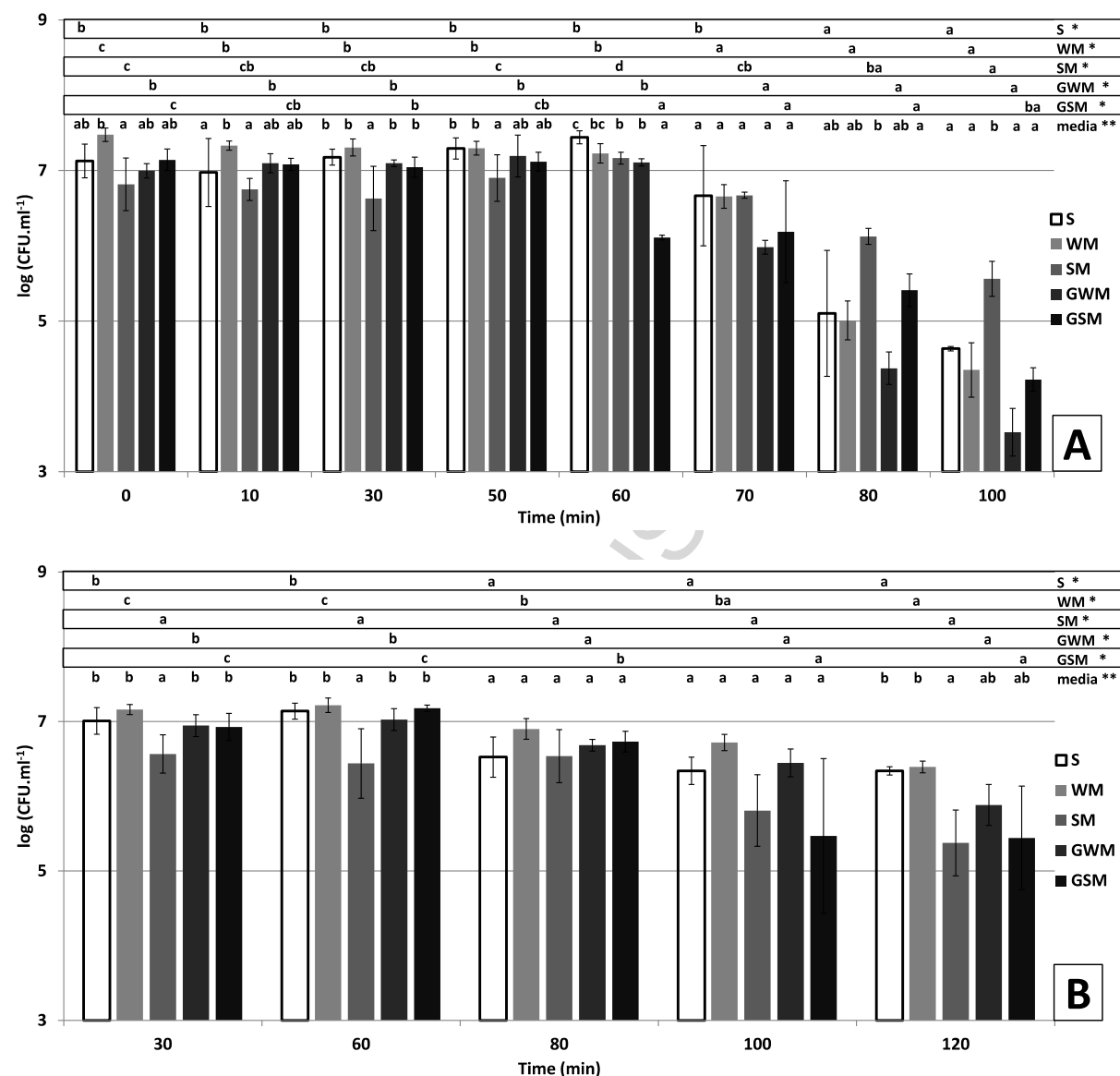
Figure 9: Buffering curves for synthetic medium, milk and rennet gel matrices during gastric phase in DIDGI[®] experiments.

Tables

Table 1: Parameters used for in-vitro dynamic digestion.

| | Input | Concentration | Quantity or flow rate | Time lapse (min) |
|--|---------------------------------|---|--------------------------|------------------|
| Stomach 37°C | Matrix | | | |
| | + | | 200 mL | 0 |
| | Artificial saliva | | | |
| | HCl | 0.5 M | pH regulation | [0;70] |
| | Pepsin | 0.5 g.L ⁻¹ (~2000 U.mL ⁻¹) | 0.4mL.min ⁻¹ | [0;90] |
| | Gastric Lipase | 6.5 g.L ⁻¹ (~200 U.mL ⁻¹) | 0.4mL.min ⁻¹ | [0;90] |
| Duodenum 37°C | Na ₂ CO ₃ | 1 M | pH regulation | |
| | Bile 40 | 40 g.L ⁻¹ | 10 mL | 0 |
| | | | 0.5mL.min ⁻¹ | [0;80] |
| | Bile 20 | 20 g.L ⁻¹ | 0.15mL.min ⁻¹ | [80;100] |
| | | | 0.2mL.min ⁻¹ | [80;220] |
| | Pancreatin | 20 g.L ⁻¹ | 5 mL | 0 |
| | | | 0.25mL.min ⁻¹ | [0;50] |
| Intestine (Jejunum and ileum) 37°C | | | 0.1mL.min ⁻¹ | [50;220] |
| | Diluent | 9.5 g.L ⁻¹ | 0.8mL.min ⁻¹ | [10;100] |
| | | | 0.5mL.min ⁻¹ | [100;250] |

Figures



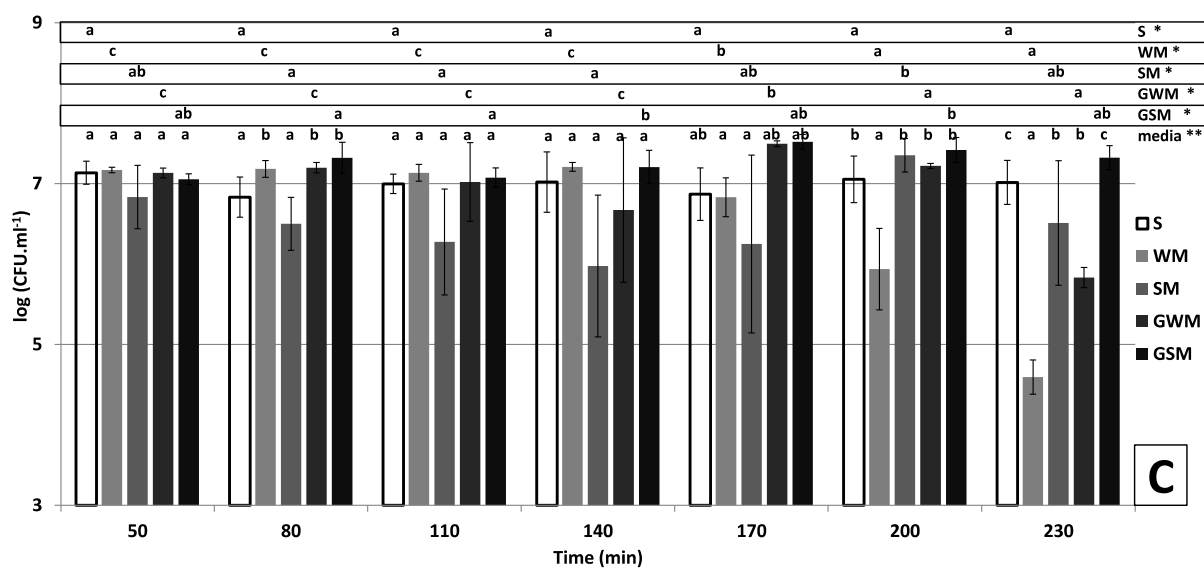
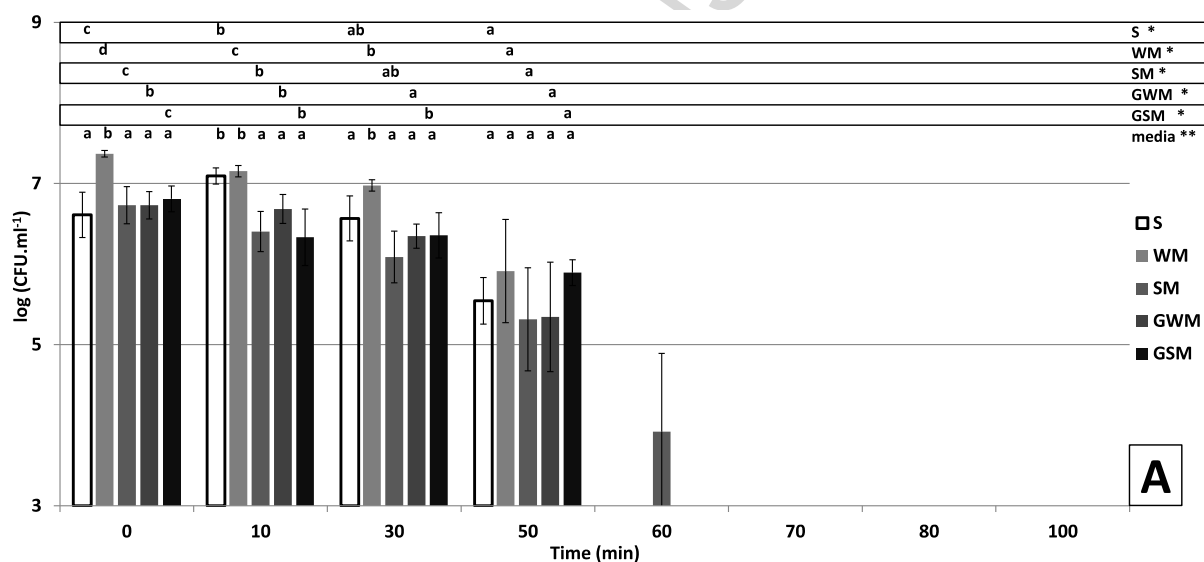


Figure 1



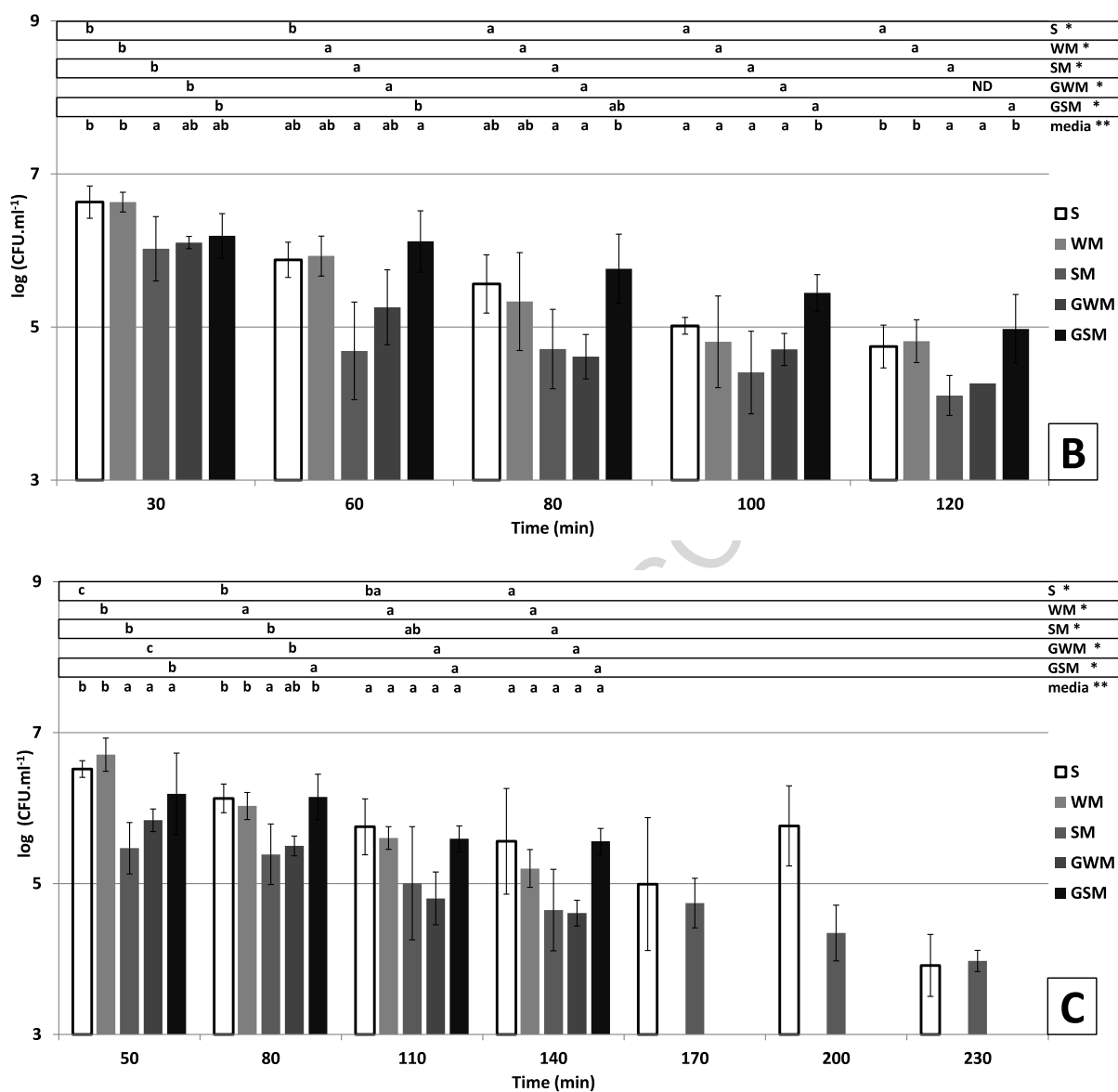


Figure 2

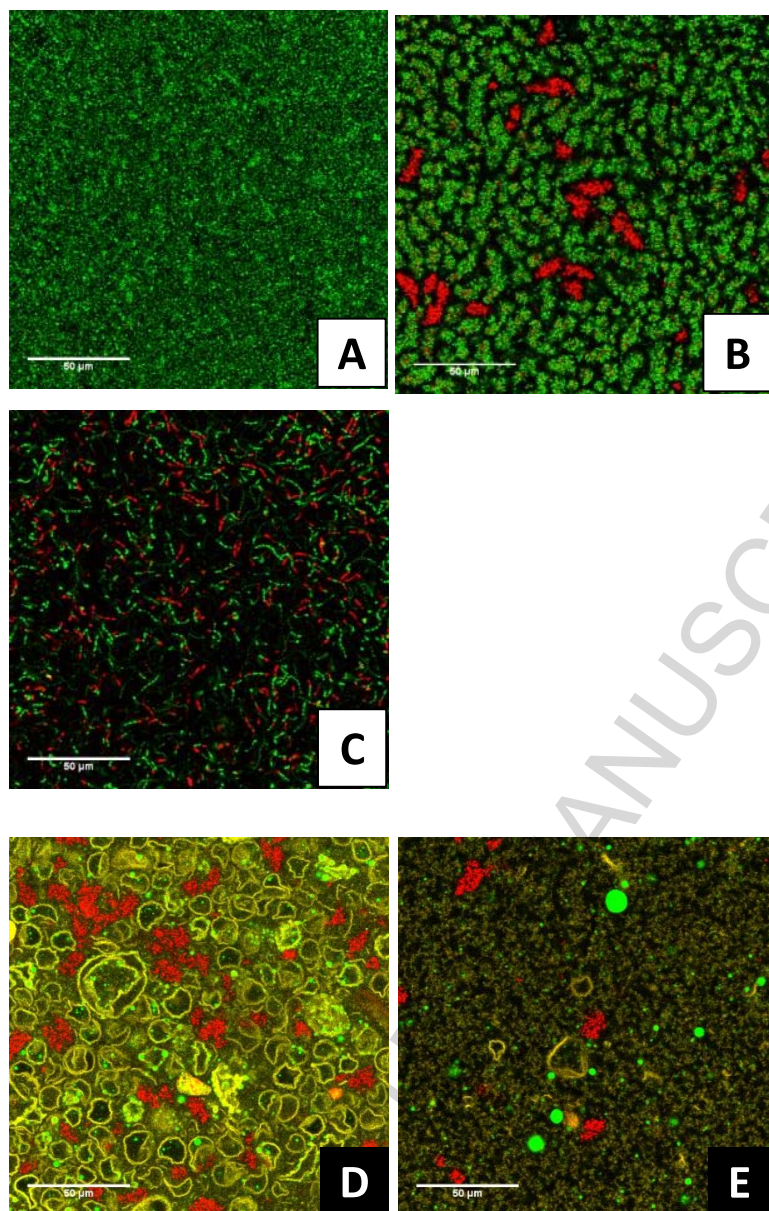


Figure 3

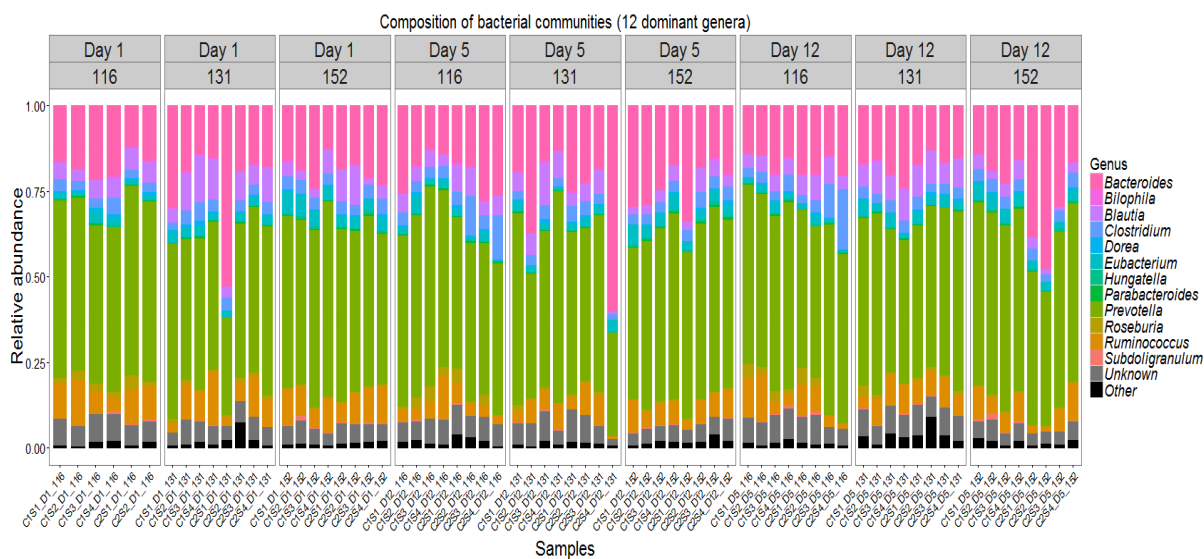


Figure 4

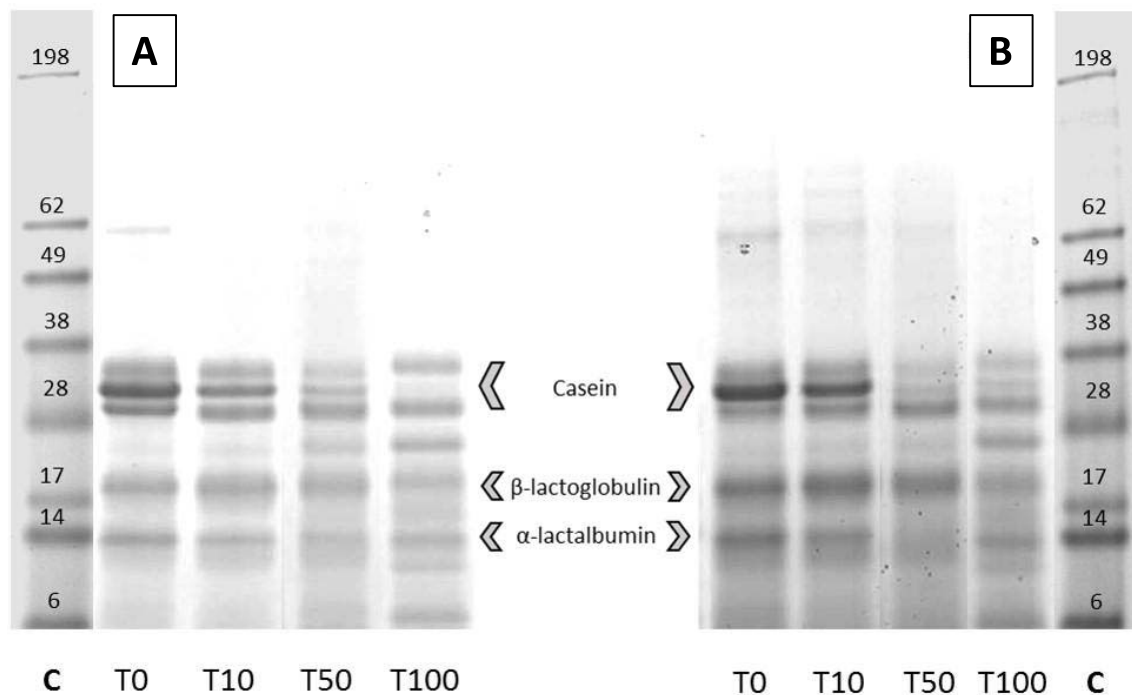


Figure 5

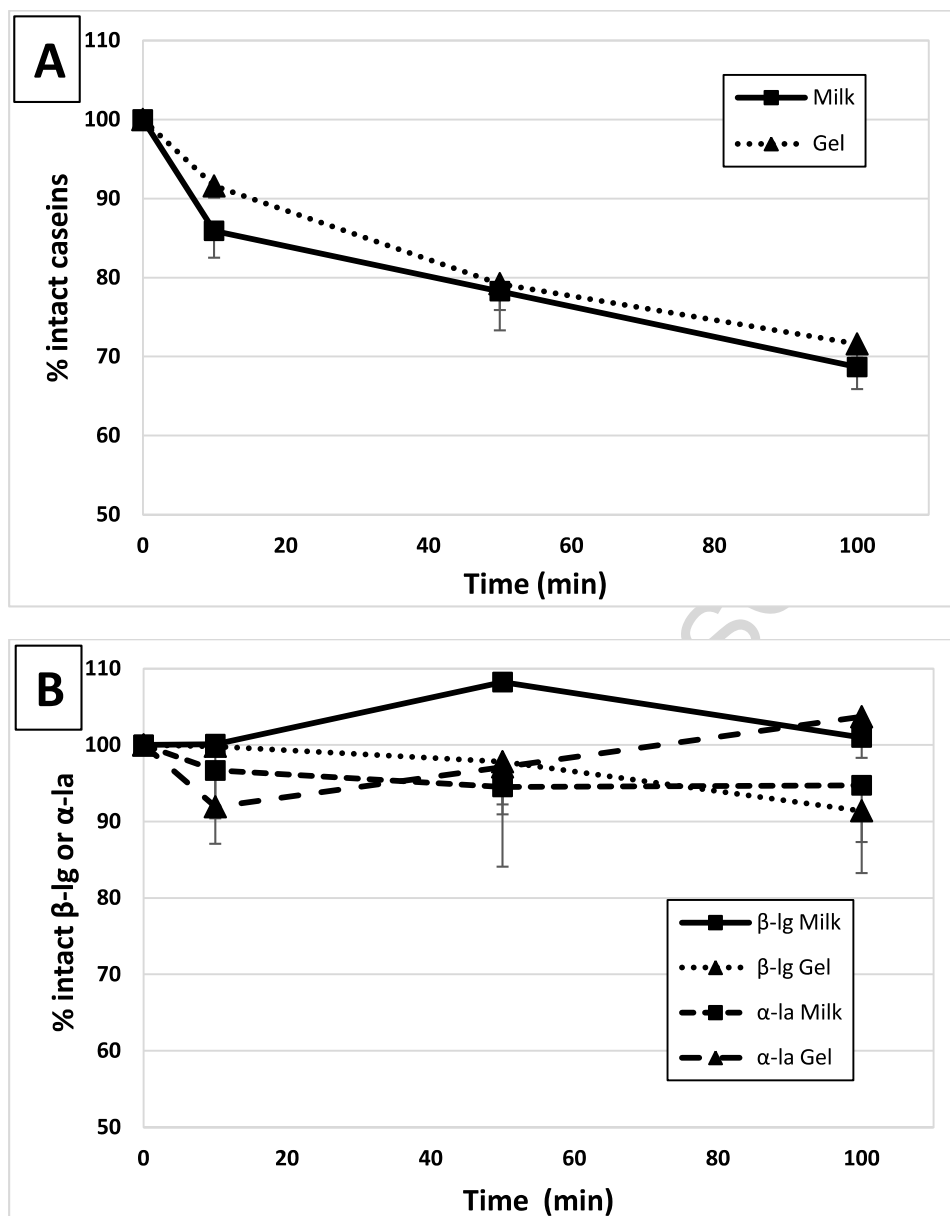


Figure 6

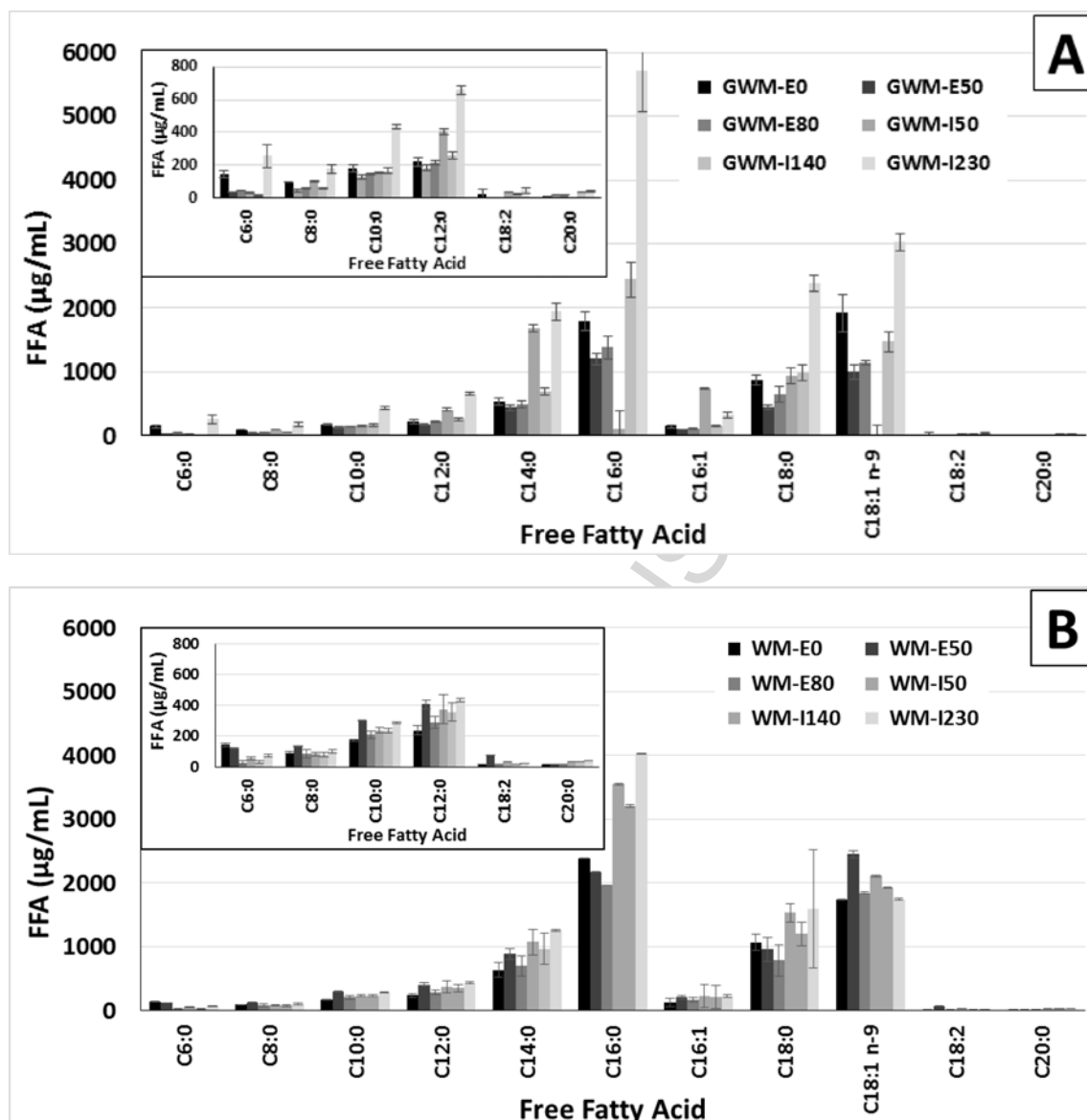


Figure 7

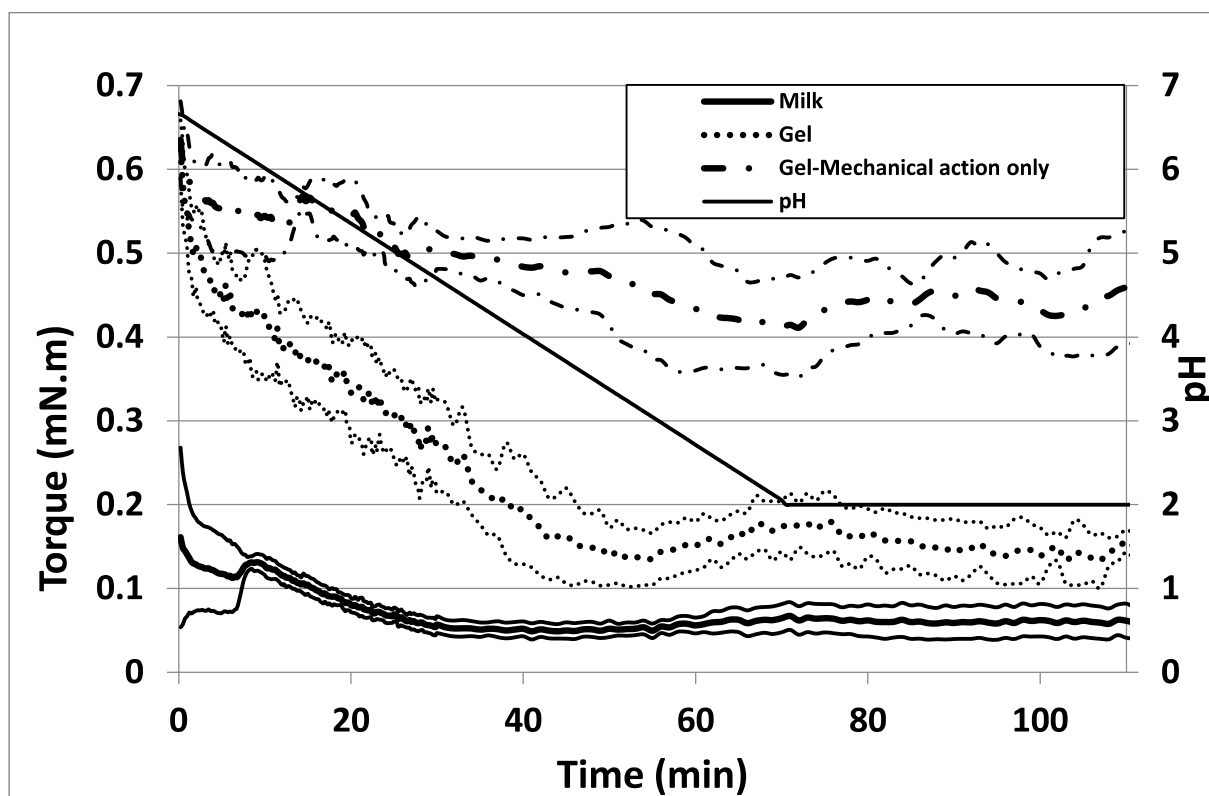


Figure 8

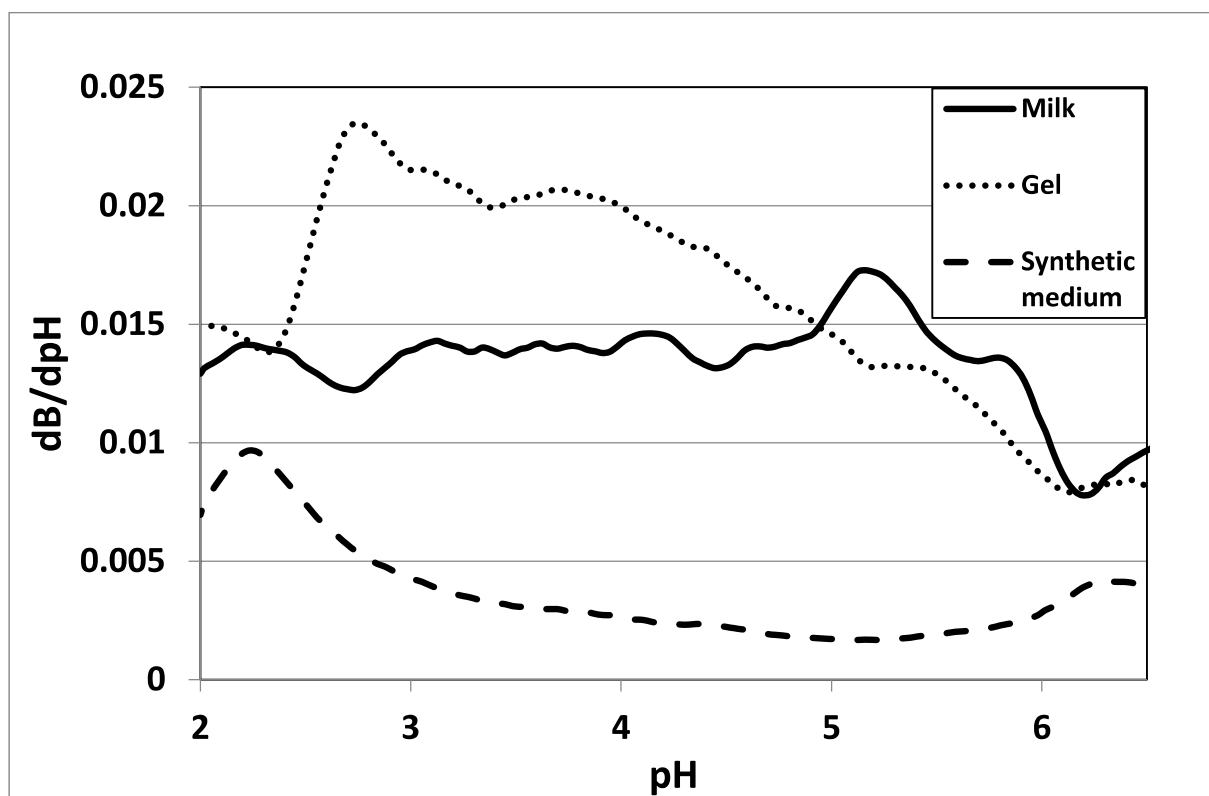
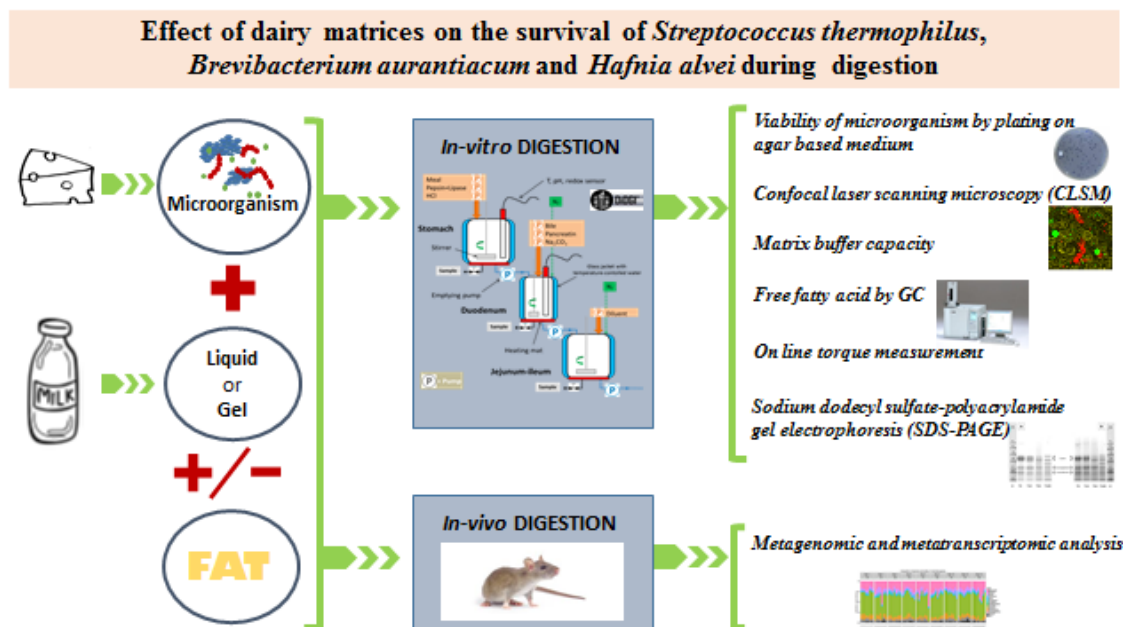


Figure 9



Graphical abstract

Highlights

- Stomach acidification was the main cause of microorganism mortality
- Dairy matrix structure and composition had no influence on microorganisms survival.
- Resistance of microorganisms to digestive stress was strain-dependent.

ACCEPTED MANUSCRIPT