SHELF LIFE OF THE WET PROTEIN PASTES UPON STORAGE

Preparation of the wet protein pastes

The wet protein pastes were produced from alfalfa, red clover and ryegrass in a “protein biorefining” pilot plant located at Foulum, research facilities from Aarhus University, during the summer season of 2017. The freshly harvested crops were processed with a screw press into a press cake and green juice. The green juice was lactic acid fermented either with a pure culture of the homofermentative lactic acid bacteria strain *Lactobacillus salivarius* (according to Santamaria-Fernandez *et al.* 2017) or through natural fermentation by the epiphytic bacteria contained in the plant material. After the precipitation of proteins because of the pH drop in the fermented juice, a wet protein paste was produced by means of decanter centrifugation.

The wet protein pastes were vacuum packed in plastic bags in order to investigate their shelf life at two conditions i.e. room temperature and in the fridge (approx. 4°C) during one-year of storage. Samples were taken at time 0 namely, when the wet protein pastes were produced, as well as after 1, 4, 8 and 12 months of storage. Four bags were prepared for each condition and storage period for the three different crops resulting in 60 bags, which were equally divided between AAU and AU.

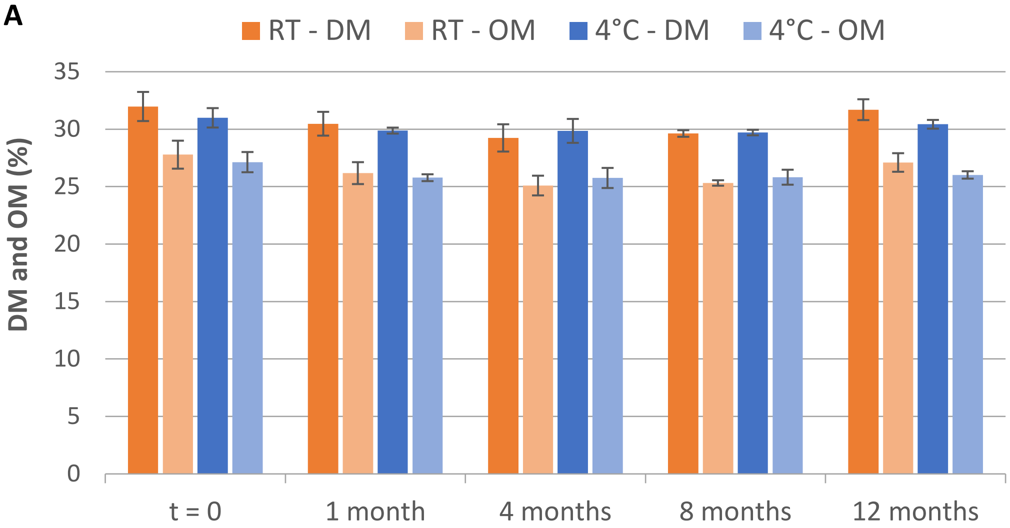
Dry matter and organic matter in the wet protein pastes

The dry matter (DM) and organic matter (OM) content were analyzed in all the wet protein paste samples in triplicates for each condition and storage period. The dry matter content was determined by drying the samples overnight at 105°C. The organic matter content was determined upon ignition of the samples at 550°C for three hours.

The DM and OM content in the wet protein pastes from alfalfa, red clover and ryegrass during storage period at room temperature (RT) or in the fridge (4°C) are shown in Fig. 1.

At time 0, the DM in the wet protein pastes was 31% for alfalfa, 29% for red clover and 28% for ryegrass while the OM in the wet protein pastes was 27% for alfalfa, 19% for red clover, and 21% for ryegrass. Therefore, a greater variability between the protein pastes produced from different crops was observed in terms of OM content at time 0, which represented 87% of DM, 64% of DM and 75% of DM in the alfalfa, red clover and ryegrass protein pastes, respectively. The relatively high content of inorganic matter in the protein pastes and differences between crops are likely because of the presence of soil in the fresh material after harvesting. In this case, red clover was probably the crop with the highest soil contamination from the harvesting. In general, the DM and OM content did not change in the wet protein pastes after one-year of storage, which suggests good preservation both at RT and at 4°C. For instance, DM losses in silage are usually related with lower quality silage, which is not the case. Minor variations observed in the data are probably because of slight differences in the composition between bags and heterogeneity of the samples.

A



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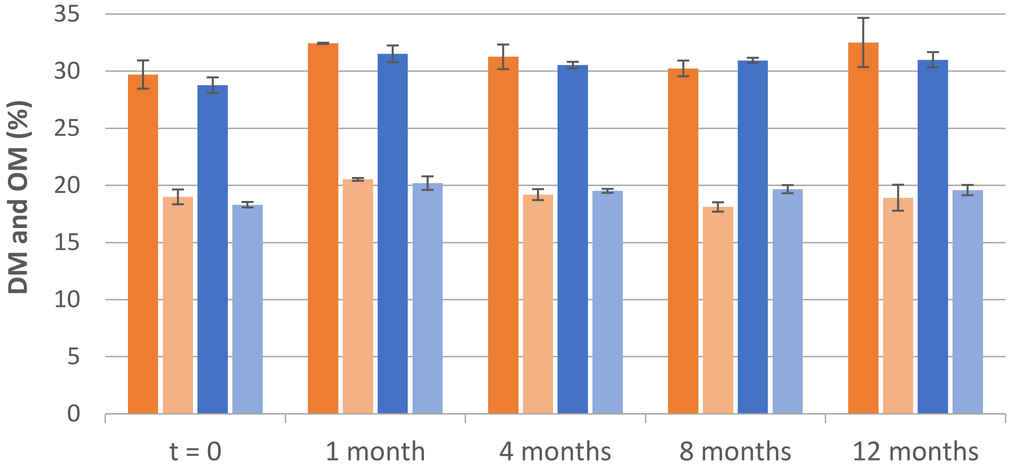

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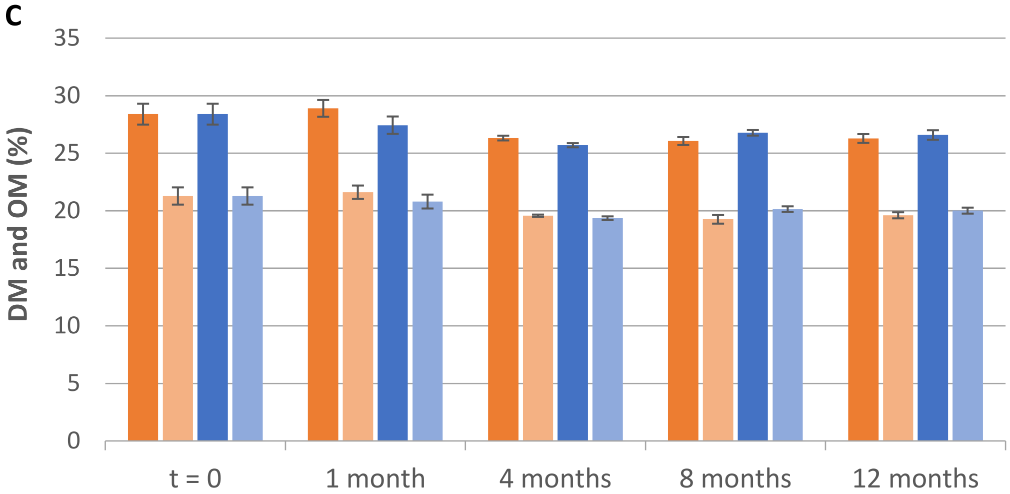

Figure 1. Dry matter (DM) and organic matter (OM) content, expressed in %, in the wet protein pastes produced from alfalfa (A), red clover (B) and ryegrass (C) during one-year of storage at room temperature (RT) or in the fridge (4°C).

pH evolution and organic acids profile in the wet protein pastes during storage

The pH was measured in all the wet protein paste samples with a pH-meter by immersion of the glass electrode in the samples.

The lactic acid content in the wet protein paste samples was determined together with the acetic acid, succinic acid, ethanol, 1,3-propanediol and free sugars (i.e. glucose, xylose-fructose, arabinose) by High Performance Liquid Chromatography (HPLC). Triplicate samples were prepared for each condition and storage period. Samples were diluted, acidified with 10% v/v of H_2SO_4 2M and centrifuged at 10,000 g for 10 minutes. Afterwards, samples were filtered through 45 µm filters into chromatography vials and analyzed by HPLC on a Dionex Ultimate 3000-LC system (Dionex Corporation, Sunnyvale, CA, USA) with an Aminex® HPX-87H column coupled to a refractive index detector. As mobile phase H_2SO_4 (4 mM) was used, with a flow rate of 0.6 ml min^{-1} at 60°C. All chromatograms were integrated using the Chromeleon software (Dionex Corporation).

The volatile fatty acids (VFAs) content in the wet protein paste samples was determined by Gas Chromatography (GC). Triplicate samples were prepared for each condition and storage period. In this case, samples were diluted, acidified with 10% v/v of HPO_4 17% and centrifuged at 10,000 g for 10 minutes. Afterwards, samples were filtered through 45 µm filters into chromatography vials and analyzed on a gas chromatograph (Shimadzu, GC-2010 Plus) equipped with an Agilent 19095F-123 capillary column of 30 m length and 0.53 mm diameter followed by flame ionization detector (FID). Nitrogen was used as carrier gas (5 ml min^{-1}) and the temperature of the detector and the injector was 240°C.

The pH was very similar (between 4.1-4.0) in all the different wet protein pastes at time 0 (Fig. 2A). However, the pH evolution was slightly different in wet protein pastes samples during the one-year storage. For instance, a stable pH was only observed in the alfalfa and ryegrass wet protein pastes stored at room temperature. However, the pH decreased to pH 3.8 in the ryegrass wet protein paste after one-year of storage in the fridge. Besides, the pH increased slightly in the alfalfa and red clover wet protein pastes stored in the fridge reaching pH 4.5-4.6 after 8 months of storage and then, pH 4.3-4.4 after one-year of storage. The greatest pH change was found in the red clover wet protein paste stored at room temperature, with a pH of 5.5 after one-year storage. Changes in pH during the storage of the wet protein pastes suggests microbial activity and are probably related with the concentration of the different organic acids in the wet protein pastes, as discussed below.

The lactic acid concentration ranged between 14-21 g kg^{-1} in the wet protein pastes at time 0 (Fig. 2B). A noticeably higher lactic acid concentration was found in the ryegrass wet protein pastes (21 g kg^{-1}) compared to alfalfa and red clover wet protein pastes (14-15 g kg^{-1}) at time 0. Such difference might be related to performance of the lactic acid fermentation in the green juice, which is influenced by the concentration of available sugars in the green juice among other factors. Changes in the lactic acid concentration were also observed during the storage of the wet protein pastes, as already showed by changes in the pH. The lactic acid concentration was rather stable in the ryegrass protein paste stored at room temperature, and so did the pH as noted above. The lactic acid concentration decreased slightly in the alfalfa and red clover wet protein pastes stored in the fridge, which is also in accordance with a slight pH increase. A significant decrease in the lactic acid concentration was found in the red clover protein paste stored at room temperature, in which any lactic acid was detected after 8 months of storage explaining thus the pH increase to 5.5. Besides, the lactic acid concentration increased slightly in the ryegrass wet protein paste stored at room temperature and more significantly in the alfalfa wet protein paste stored at room temperature, for which a lactic acid doubled from 15 g kg^{-1} to 32 g kg^{-1} after one-year of storage.

The acetic acid concentration was between 6-8 g kg^{-1} in the wet protein pastes at time 0 (Fig. 2C). The presence of acetic acid in the wet protein pastes indicates the activity of hetero-fermentative lactic acid bacteria during the fermentation of the green juice, even if a homo-fermentative strain was utilized in the process. The acetic acid concentration increased in most protein paste samples during the storage period and a maximum concentration of about 14 g kg^{-1} was detected in the alfalfa wet protein pastes stored at room temperature after one-year of storage. Therefore, hetero-fermentative bacteria were active during the storage of the protein pastes at room temperature and at 4°C.

In addition, low concentrations of volatile fatty acids (VFAs) were detected in some of the wet protein paste samples during the storage period, being propionic acid the most recurring VFA in the samples especially during storage at room temperature (Tables 1-3). Mostly, a greater presence of VFAs was observed during storage of the wet protein pastes at room temperature compared to storage in the fridge (4°C). In particular, a high concentration of butyric acid (about 8 g kg^{-1}) was observed in the red clover protein paste stored at room temperature suggesting that the lactic acid was probably consumed for the production of butyric acid in this case.

Some succinic acid, ethanol and 1,3-propanediol were also detected in the wet protein pastes samples during the storage period (Tables 1-3). Succinic acid concentrations up to 9 g kg^{-1} were found in the alfalfa wet protein paste stored at room temperature and up to about 5 g kg^{-1} were found in the ryegrass wet protein paste stored both at room temperature and at 4°C. Besides, ethanol concentrations up to 4 g kg^{-1} were found in alfalfa wet protein paste stored at room temperature and up to 17 g kg^{-1} were found in the red clover wet protein paste stored at room temperature.

The presence of a mixture of lactic acid, acetic acid, succinic acid and ethanol in the wet protein paste samples suggests the occurrence of mixed acid fermentation, which is common in bacteria under anaerobic conditions. Lactic acid bacteria are also able to produce a mixture of lactic acid, formic acid, acetic acid, propionic acid and succinic acid, especially in limiting substrate conditions. Indeed, very low concentration of free sugars (i.e. glucose, fructose/xylose and arabinose) were detected in the wet protein pastes during the storage period (Tables 1-3).

No trend was observed in the organic acids profile during the one-year storage of the different wet protein paste samples but changes in the pH and concentration of acids suggests a relatively high microbial activity especially in the samples stored at room temperature.

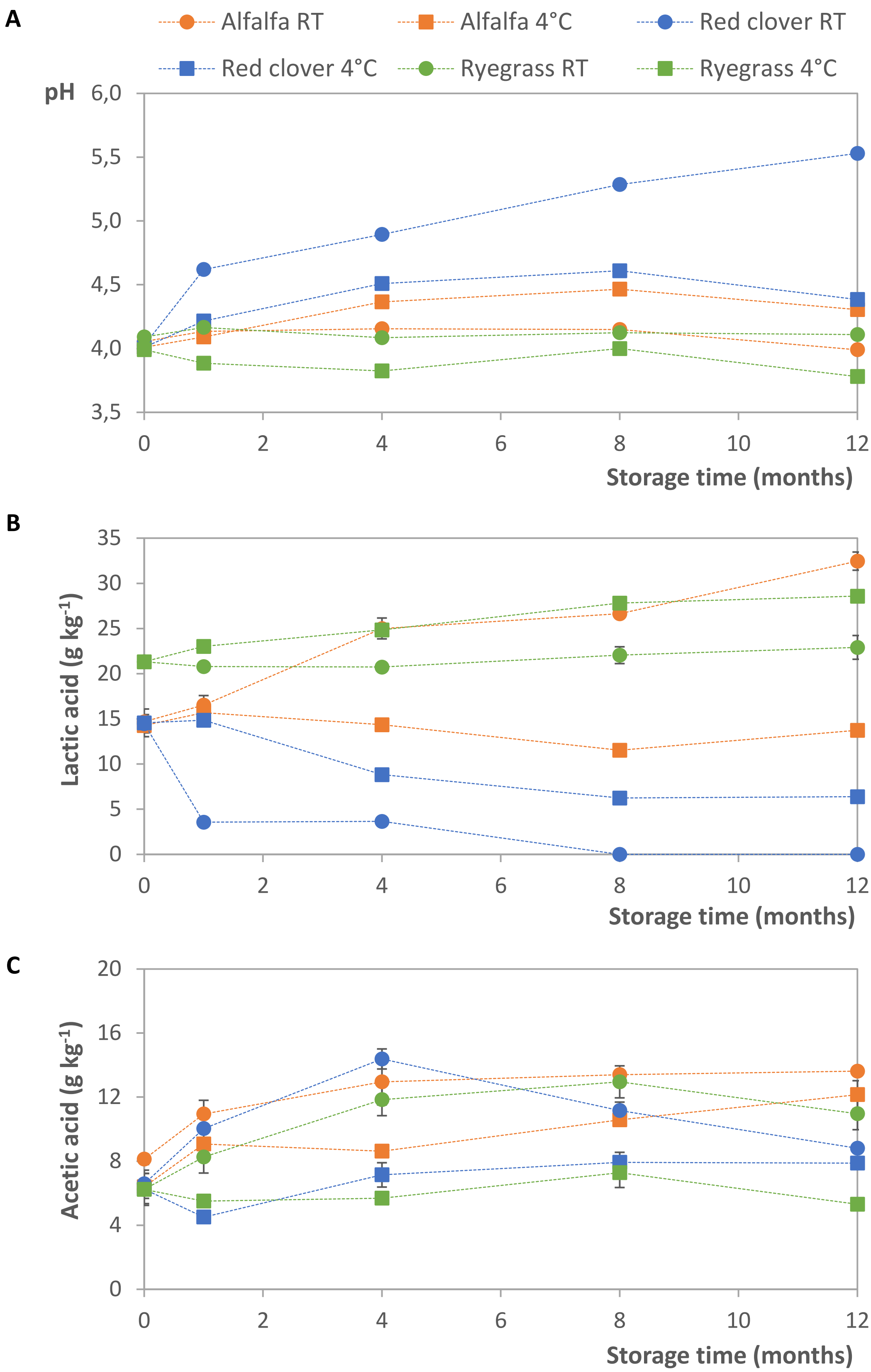

Figure 2. Evolution of pH (A), lactic acid (B) and acetic acid (C) in the wet protein pastes produced from alfalfa, red clover and ryegrass during one-year storage at room temperature (RT) or in the fridge (4°C).

Microbial growth in the wet protein pastes during storage

The microbial growth was assessed in the wet protein paste samples during the storage period at room temperature or in the fridge (4°C). A dilution series was prepared for each sample in duplicates (i.e. one dilution series per sample bag) and then, the diluted samples were spread in plates containing solid growth media. Three different growth media were utilized in duplicates for each diluted sample: LB to assess the growth of bacteria, MRS to assess the growth of *Lactobacilli* and YPD to assess the growth of yeast. The different plates were incubated at 38°C for 72 hours and afterwards, the number of colonies was counted.

The growth of bacteria, *Lactobacilli* and yeast in the wet protein paste samples at time 0, expressed in terms of colony-forming units (Cfu) per kg of sample, is shown in Fig. 3. In general, large numbers of bacteria, *Lactobacilli* and yeast were found in the wet protein pastes at time 0. However, the microbial growth varied significantly between protein pastes produced from the three different crops. Indeed, red clover wet protein paste presented by far the highest microbial growth while ryegrass wet protein pastes presented the lowest microbial growth. Most microorganisms present in the wet protein pastes come from the fresh plant material and therefore, differences in the microbial growth between protein pastes from the different crops are likely to be due to differences in the microbial community in the fresh plant material. Red clover wet protein pastes presented also the highest content of inorganic material, probably because of the presence of soil and therefore, a large microbial growth could also be related to soil contamination.

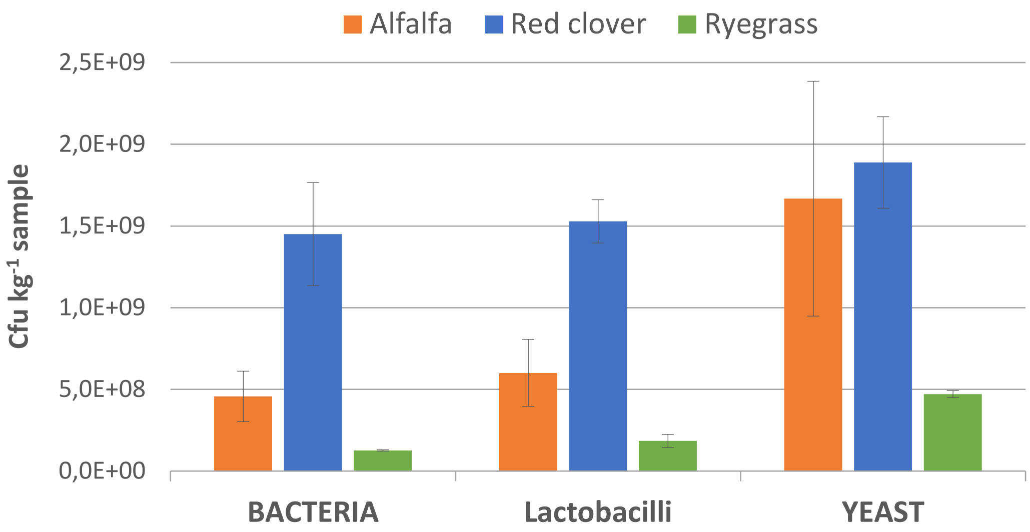

Figure 3. Microbial growth, expressed in terms of Cfu per kg sample, in the wet protein pastes produced from alfalfa, red clover and ryegrass at time 0.

On the other hand, the microbial growth in the wet protein paste samples during the one-year storage at room temperature or in the fridge (4°C) is summarized in Fig. 4. In general, large variability in the microbial growth was observed between duplicate samples and no clear trend was noted during the one-year storage of the wet protein pastes. However, some key points can be obtained from the results. Firstly, the microbial growth was significantly reduced after one month of storage both, at room temperature and in the fridge (4°C) in all the wet protein pastes samples. Moreover, the microbial growth generally decreased in the wet protein paste samples during the storage period and after one year of storage, the microbial growth was significantly reduced i.e. between 2-4 orders of magnitude in most cases. Besides, higher microbial growth was often observed in the protein paste samples stored in the fridge (4°C) suggesting that microorganisms were better preserved in the protein pastes after storage in the fridge rather than at room temperature. It is likely that the increased microbial activity observed in the protein pastes stored at room temperature resulted in a faster depletion of nutrients, especially C sources and therefore, microorganisms reached their death phase earlier than when the protein pastes were stored in the fridge (4°C).

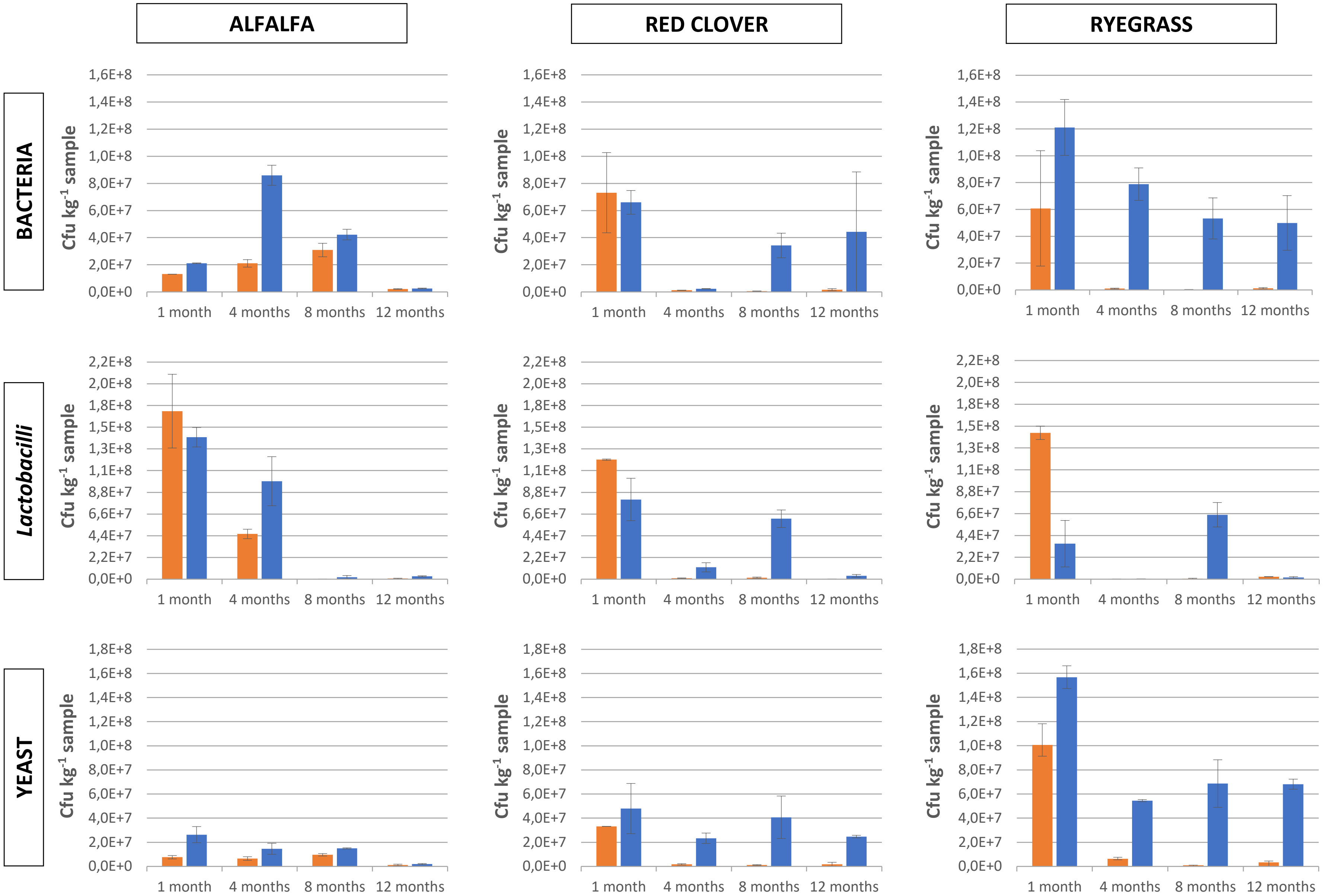

Figure 4. Microbial growth, expressed in terms of Cfu per kg sample, in the wet protein pastes produced from alfalfa, red clover and ryegrass during one-year storage at room temperature (ORANGE) or in the fridge (BLUE).

Table 1. pH and concentration of different organic acids and free sugars in the alfalfa wet protein pastes during one-year of storage at room temperature or in the fridge (4°C). Standard deviation shown in brackets. Red indicates below detection limit.

	Room temperature					**Fridge (4°C)**				
	t = 0	*1 month*	*4 months*	*8 months*	*12 months*	*t = 0*	*1 month*	*4 months*	*8 months*	*12 months*
pH	4.1 (0.0)	4.1 (0.1)	4.2 (0.0)	4.2 (0.0)	4.0 (0.0)	4.0 (0.0)	4.1 (0.0)	4.4 (0.0)	4.5 (0.0)	4.3 (0.0)
Lactic acid (g/kg)	14.7 (0.5)	16.5 (1.0)	25.0 (1.2)	26.6 (0.1)	32.5 (1.0)	14.3 (0.6)	15.7 (0.7)	14.3 (0.4)	11.5 (0.6)	13.7 (0.3)
Acetic acid (g/kg) HPLC	3.4 (0.0)	10.9 (0.6)	12.5 (0.5)	13.6 (0.1)	14.0 (0.6)	2.3 (0.0)	5.0 (0.1)	8.6 (0.0)	10.7 (0.1)	11.7 (0.2)
Acetic acid (g/kg) GC	8.1 (0.3)	10.9 (0.9)	12.9 (1.4)	13.4 (0.1)	13.6 (0.1)	6.4 (1.0)	9.1 (0.8)	8.6 (0.2)	10.6 (0.3)	12.2 (0.9)
Propionic acid (g/kg)	3.0 (0.3)	2.7 (1.0)	2.0 (0.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	2.4 (0.1)	2.1 (0.1)	0.0 (0.0)	0.0 (0.0)
Iso-butyric acid (g/kg)	1.7 (0.2)	1.5 (0.6)	0.9 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Butyric acid (g/kg)	1.7 (0.2)	1.8 (0.7)	1.0 (0.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	1.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Iso-valeric acid (g/kg)	0.5 (0.1)	0.6 (0.2)	0.3 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.4 (0.0)	0.0 (0.0)	0.0 (0.0)
Valeric acid (g/kg)	0.5 (0.1)	0.6 (0.2)	0.4 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.4 (0.0)	0.0 (0.0)	0.0 (0.0)
Succinic acid (g/kg)	0.0 (0.0)	7.0 (0.3)	8.8 (0.3)	8.6 (0.1)	8.3 (0.2)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Ethanol (g/kg)	0.0 (0.0)	2.9 (0.3)	3.5 (0.6)	1.9 (0.1)	3.6 (0.7)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	1.0 (0.2)
1,3 Propanediol (g/kg)	0.0 (0.0)	0.4 (0.0)	0.5 (0.0)	0.0 (0.0)	0.5 (0.1)	0.0 (0.0)	0.0 (0.0)	0.8 (0.2)	1.4 (0.0)	1.7 (0.1)
Glucose (g/kg)	1.7 (0.1)	0.2 (0.0)	0.1 (0.0)	0.0 (0.0)	0.0 (0.0)	1.7 (0.1)	1.4 (0.1)	0.5 (0.0)	0.2 (0.0)	0.0 (0.0)
Fructose/xylose (g/kg)	1.2 (0.1)	1.4 (0.1)	1.8 (0.1)	2.0 (0.0)	1.9 (0.3)	1.1 (0.1)	1.4 (0.4)	1.6 (0.1)	1.5 (0.0)	1.6 (0.1)
Arabinose (g/kg)	0.0 (0.0)	0.3 (0.0)	0.4 (0.0)	0.3 (0.1)	0.2 (0.0)	0.0 (0.0)	0.0 (0.0)	0.9 (0.0)	1.1 (0.0)	1.1 (0.0)

Table 2. pH and concentration of different organic acids and free sugars in the red clover wet protein pastes during one-year of storage at room temperature or in the fridge (4°C). Standard deviation shown in brackets. Red indicates below detection limit.

	Room temperature					**Fridge (4°C)**				
	t = 0	*1 month*	*4 months*	*8 months*	*12 months*	*t = 0*	*1 month*	*4 months*	*8 months*	*12 months*
pH	4.0 (0.0)	4.6 (0.0)	4.9 (0.0)	5.3 (0.1)	5.5 (0.0)	4.0 (0.0)	4.2 (0.0)	4.5 (0.1)	4.6 (0.0)	4.4 (0.0)
Lactic acid (g/kg)	14.5 (1.0)	3.6 (0.1)	3.6 (0.0)	0.0 (0.0)	0.0 (0.0)	14.6 (1.5)	14.9 (0.0)	8.8 (0.3)	6.2 (0.4)	6.4 (0.2)
Acetic acid (g/kg) HPLC	2.4 (0.2)	9.9 (0.2)	13.3 (0.1)	12.0 (0.1)	9.8 (0.1)	2.2 (0.2)	3.3 (0.1)	6.5 (0.1)	7.7 (0.1)	8.7 (0.1)
Acetic acid (g/kg) GC	6.6 (0.6)	10.0 (0.7)	14.4 (0.6)	11.2 (0.5)	8.8 (0.0)	6.2 (0.6)	4.5 (0.4)	7.1 (0.8)	7.9 (0.6)	7.9 (0.0)
Propionic acid (g/kg)	0.0 (0.0)	1.8 (0.0)	2.0 (0.0)	3.8 (0.2)	3.5 (0.3)	0.0 (0.0)	0.0 (0.0)	2.5 (0.9)	2.0 (0.1)	1.6 (0.0)
Iso-butyric acid (g/kg)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	1.5 (0.1)	1.7 (0.3)	0.0 (0.0)	0.0 (0.0)	1.9 (0.6)	0.0 (0.0)	0.0 (0.0)
Butyric acid (g/kg)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	4.8 (0.8)	7.7 (1.7)	0.0 (0.0)	0.0 (0.0)	2.0 (0.7)	1.0 (0.1)	0.0 (0.0)
Iso-valeric acid (g/kg)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	1.4 (0.4)	2.7 (0.7)	0.0 (0.0)	0.0 (0.0)	0.6 (0.2)	0.0 (0.0)	0.0 (0.0)
Valeric acid (g/kg)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.9 (0.1)	1.8 (0.2)	0.0 (0.0)	0.0 (0.0)	0.6 (0.2)	0.0 (0.0)	0.0 (0.0)
Succinic acid (g/kg)	0.0 (0.0)	1.4 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.4 (0.1)
Ethanol (g/kg)	0.0 (0.0)	1.8 (0.2)	0.0 (0.0)	9.5 (1.1)	16.6 (2.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	3.5 (0.3)	0.0 (0.0)
1,3 Propanediol (g/kg)	0.0 (0.0)	0.7 (0.1)	0.7 (0.0)	2.9 (0.0)	3.6 (0.7)	0.0 (0.0)	0.0 (0.0)	0.8 (0.2)	0.8 (0.1)	1.7 (0.1)
Glucose (g/kg)	1.3 (0.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	1.2 (0.0)	0.5 (0.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Fructose/xylose (g/kg)	0.7 (0.1)	0.8 (0.1)	1.1 (0.0)	1.1 (0.0)	1.3 (0.2)	0.8 (0.1)	0.0 (0.0)	1.1 (0.0)	0.6 (0.1)	0.8 (0.0)
Arabinose (g/kg)	0.0 (0.0)	1.3 (0.0)	1.2 (0.0)	0.8 (0.1)	1.2 (0.1)	0.0 (0.0)	0.0 (0.0)	1.1 (0.0)	1.0 (0.0)	1.2 (0.0)

Table 3. pH and concentration of different organic acids and free sugars in the ryegrass wet protein pastes during one-year of storage at room temperature or in the fridge (4°C). Standard deviation shown in brackets. Red indicates below detection limit.

	Room temperature					**Fridge (4°C)**				
	t = 0	*1 month*	*4 months*	*8 months*	*12 months*	*t = 0*	*1 month*	*4 months*	*8 months*	*12 months*
pH	4.1 (0.1)	4.2 (0.0)	4.1 (0.0)	4.1 (0.0)	4.1 (0.0)	4.0 (0.0)	3.9 (0.0)	3.8 (0.0)	4.0 (0.0)	3.8 (0.0)
Lactic acid (g/kg)	21.3 (0.2)	20.8 (0.5)	20.7 (0.1)	22.0 (0.9)	22.9 (1.3)	21.3 (0.2)	23.0 (0.5)	24.8 (0.2)	27.8 (0.1)	28.6 (0.4)
Acetic acid (g/kg) HPLC	5.9 (0.2)	7.0 (0.1)	10.4 (0.1)	11.7 (0.2)	13.3 (0.0)	5.9 (0.2)	4.7 (0.1)	5.1 (0.1)	5.7 (0.1)	5.6 (0.1)
Acetic acid (g/kg) GC	6.2 (0.1)	8.3 (1.3)	11.8 (2.1)	13.0 (2.2)	11.0 (0.1)	6.2 (0.1)	5.5 (0.2)	5.7 (0.3)	7.3 (0.9)	5.3 (0.1)
Propionic acid (g/kg)	1.5 (0.0)	1.6 (0.1)	2.0 (0.2)	2.3 (0.2)	1.8 (0.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Iso-butyric acid (g/kg)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Butyric acid (g/kg)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	1.0 (0.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Iso-valeric acid (g/kg)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Valeric acid (g/kg)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Succinic acid (g/kg)	4.5 (0.2)	4.5 (0.3)	3.7 (0.0)	3.7 (0.1)	3.7 (0.3)	4.5 (0.2)	3.0 (0.1)	3.4 (0.0)	3.5 (0.0)	2.6 (0.6)
Ethanol (g/kg)	0.0 (0.0)	0.0 (0.0)	0.8 (0.2)	1.2 (0.2)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
1,3 Propanediol (g/kg)	0.4 (0.1)	0.7 (0.0)	0.9 (0.0)	1.0 (0.0)	2.4 (0.1)	0.4 (0.1)	0.0 (0.0)	0.3 (0.0)	0.0 (0.0)	0.0 (0.0)
Glucose (g/kg)	0.3 (0.1)	0.3 (0.0)	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)	0.3 (0.1)	0.3 (0.0)	0.2 (0.0)	0.4 (0.1)	0.5 (0.0)
Fructose/xylose (g/kg)	1.2 (0.0)	1.3 (0.1)	1.5 (0.0)	1.6 (0.0)	1.7 (0.1)	1.2 (0.0)	1.2 (0.0)	1.3 (0.0)	1.6 (0.0)	1.9 (0.0)
Arabinose (g/kg)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.2 (0.1)

Table 4. Microbial growth observed in the alfalfa wet protein pastes during one-year of storage at room temperature or in the fridge (4°C).

	Storage time (months)	**Room temperature**	**Fridge (4 °C)**
Bacteria	0	$4.6\ 10^{8}$ ($1.5\ 10^{7}$)	$4.6\ 10^{8}$ ($1.5\ 10^{8}$)
	1	$1.3\ 10^{7}$ ($1.1\ 10^{4}$)	$2.1\ 10^{7}$ ($2.5\ 10^{5}$)
	4	$2.1\ 10^{7}$ ($2.8\ 10^{6}$)	$8.6\ 10^{7}$ ($7.4\ 10^{6}$)
	8	$3.1\ 10^{7}$ ($5.0\ 10^{6}$)	$4.2\ 10^{7}$ ($3.9\ 10^{6}$)
	12	$2.2\ 10^{6}$ ($2.3\ 10^{5}$)	$2.6\ 10^{6}$ ($2.6\ 10^{5}$)
Lactobacilli	0	$6.0\ 10^{8}$ ($2.0\ 10^{8}$)	$6.0\ 10^{8}$ ($2.0\ 10^{8}$)
	1	$1.7\ 10^{8}$ ($3.7\ 10^{7}$)	$1.4\ 10^{8}$ ($9.9\ 10^{6}$)
	4	$4.6\ 10^{7}$ ($4.8\ 10^{6}$)	$9.9\ 10^{7}$ ($2.5\ 10^{7}$)
	8	$5.7\ 10^{5}$ ($1.3\ 10^{5}$)	$2.0\ 10^{7}$ ($1.7\ 10^{7}$)
	12	$6.5\ 10^{5}$ ($3.2\ 10^{5}$)	$2.9\ 10^{6}$ ($5.3\ 10^{5}$)
Yeast	0	$1.7\ 10^{9}$ ($7.2\ 10^{8}$)	$1.7\ 10^{9}$ ($7.2\ 10^{8}$)
	1	$7.6\ 10^{6}$ ($1.4\ 10^{6}$)	$2.6\ 10^{7}$ ($6.7\ 10^{6}$)
	4	$6.6\ 10^{6}$ ($1.5\ 10^{6}$)	$1.5\ 10^{7}$ ($4.6\ 10^{6}$)
	8	$9.6\ 10^{6}$ ($1.0\ 10^{6}$)	$1.5\ 10^{7}$ ($4.7\ 10^{5}$)
	12	$1.2\ 10^{6}$ ($6.4\ 10^{5}$)	$2.0\ 10^{6}$ ($4.4\ 10^{5}$)

Table 5. Microbial growth observed in the red clover wet protein pastes during one-year of storage at room temperature or in the fridge (4°C).

	Storage time (months)	**Room temperature**	**Fridge (4 °C)**
Bacteria	0	$1.4\ 10^{9}$ ($3.2\ 10^{8}$)	$1.4\ 10^{9}$ ($3.2\ 10^{8}$)
	1	$7.3\ 10^{7}$ ($3.0\ 10^{7}$)	$6.6\ 10^{7}$ ($8.7\ 10^{6}$)
	4	$1.3\ 10^{6}$ ($7.4\ 10^{4}$)	$2.4\ 10^{6}$ ($1.5\ 10^{5}$)
	8	$5.9\ 10^{5}$ ($1.1\ 10^{5}$)	$3.4\ 10^{7}$ ($9.1\ 10^{6}$)
	12	$1.7\ 10^{6}$ ($7.8\ 10^{5}$)	$4.4\ 10^{7}$ ($4.4\ 10^{7}$)
Lactobacilli	0	$1.5\ 10^{9}$ ($1.3\ 10^{8}$)	$1.5\ 10^{9}$ ($1.3\ 10^{8}$)
	1	$1.2\ 10^{8}$ ($6.7\ 10^{5}$)	$8.1\ 10^{7}$ ($2.1\ 10^{7}$)
	4	$9.7\ 10^{5}$ ($3.0\ 10^{5}$)	$1.2\ 10^{7}$ ($4.6\ 10^{6}$)
	8	$1.5\ 10^{6}$ ($6.1\ 10^{5}$)	$6.1\ 10^{7}$ ($8.9\ 10^{6}$)
	12	$2.3\ 10^{5}$ ($4.1\ 10^{4}$)	$3.4\ 10^{6}$ ($1.3\ 10^{6}$)
Yeast	0	$1.9\ 10^{9}$ ($2.8\ 10^{8}$)	$1.9\ 10^{9}$ ($2.8\ 10^{8}$)
	1	$3.3\ 10^{7}$ ($0.0\ 10^{0}$)	$4.8\ 10^{7}$ ($2.1\ 10^{7}$)
	4	$1.7\ 10^{6}$ ($5.5\ 10^{5}$)	$2.3\ 10^{7}$ ($4.3\ 10^{6}$)
	8	$1.2\ 10^{6}$ ($2.8\ 10^{5}$)	$4.1\ 10^{7}$ ($1.8\ 10^{7}$)
	12	$1.9\ 10^{6}$ ($1.5\ 10^{6}$)	$2.5\ 10^{7}$ ($1.1\ 10^{7}$)

Table 6. Microbial growth observed in the ryegrass wet protein pastes during one-year of storage at room temperature or in the fridge (4°C).

	Storage time (months)	**Room temperature**	**Fridge (4 °C)**
Bacteria	0	1.3 10^8 (4.0 10^6)	1.3 10^8 (4.0 10^6)
	1	6.1 10^7 (4.3 10^7)	1.2 10^8 (2.1 10^7)
	4	1.0 10^6 (3.4 10^5)	7.9 10^7 (1.2 10^7)
	8	2.6 10^5 (7.4 10^4)	5.3 10^7 (1.5 10^7)
	12	1.3 10^6 (4.8 10^5)	5.0 10^7 (2.0 10^7)
Lactobacilli	0	1.8 10^8 (4.0 10^7)	1.8 10^8 (4.0 10^7)
	1	1.5 10^8 (6.7 10^6)	3.6 10^7 (2.3 10^7)
	4	1.4 10^5 (8.7 10^4)	3.4 10^5 (6.7 10^4)
	8	5.0 10^5 (4.6 10^5)	6.5 10^7 (1.2 10^7)
	12	2.5 10^6 (1.3 10^5)	1.7 10^6 (9.2 10^5)
Yeast	0	4.7 10^8 (2.1 10^7)	4.7 10^8 (2.1 10^7)
	1	1.0 10^8 (1.7 10^7)	1.6 10^8 (9.4 10^6)
	4	6.4 10^6 (1.2 10^6)	5.4 10^7 (8.7 10^5)
	8	1.0 10^6 (2.0 10^4)	6.9 10^7 (2.0 10^7)
	12	3.4 10^6 (1.1 10^6)	6.8 10^7 (4.2 10^6)

Protein and Amino acid composition in the protein pastes

The content of selected amino acids in the protein paste from red clover was analysed using HPLC. Included were the samples taken at time of production and after 12 months of storage at either room temperature or 4°C. The results expressed as % of crude protein(CP) can be seen in Figure 5, which 1 also reports the total nitrogen content of the protein pastes. Figure 2 despicts the composition as g/kg dry matter (DM).

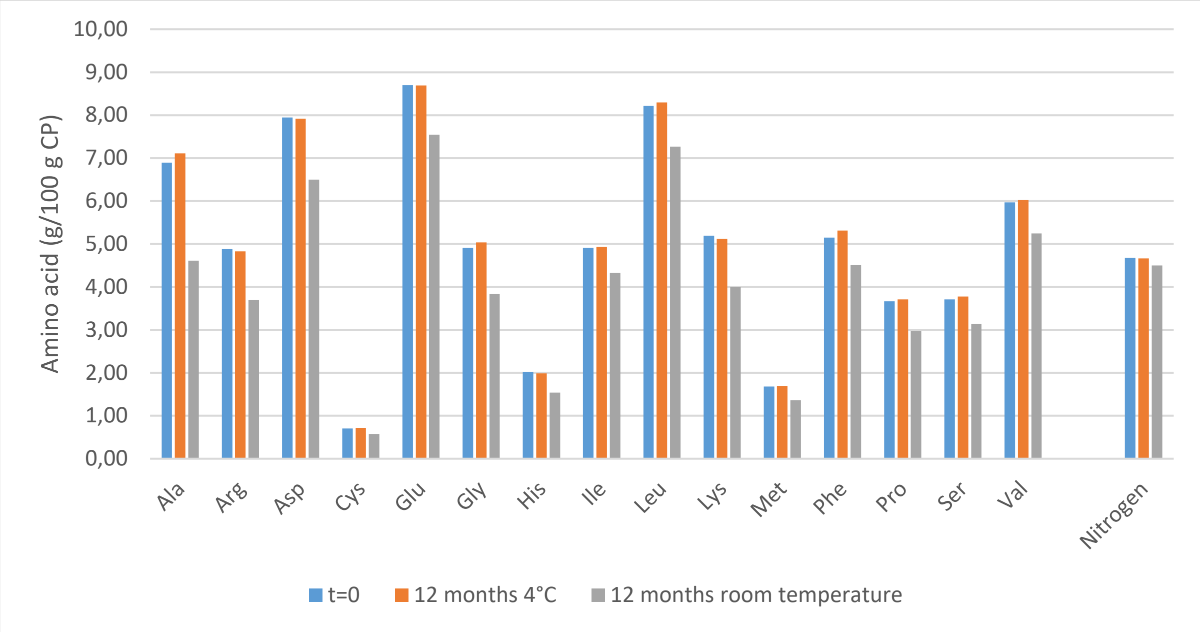

Figure 5. Amino acid composition and nitrogen content of red clover protein paste at time of production and after 12 months of storage either at room temperature or at 4°C. Amino acid results are in g/100 g CP, nitrogen in g/100 g DM.

The protein paste has a nitrogen content of 4.6 g/100 g DM corrosponding to 28.75 % protein. This was a low content compared to earlier findings. The content of nitrogen remained contant after storage for 12 months at 4°C. At room temperature, the nitrogen content decreased slightly. Alanine, aspartic acid, glutamic acid and leucine were the amino acids with the highest content,whereas the contents of the suplhur-containing amino acids, methionine and cysteine, were low consistent with earlier findings [1]. In general, the contents of all amino acids were low compared to earlier findings on red clover [1] indicating that a larger proportion of the protein paste consisted of non-protein nitrogen than what was previoiusly found. A high content of non-protein nitrogen would also explain the low total nitrogen content found in the protein paste. Storage at room temperature for 12 months clearly reduced the content of all amino acids in the protein paste, suggesting a degradation of both intact amino acids but also of of free amino acids, resulting in a larger proportion of the CP being constituted by other nitrogen components.

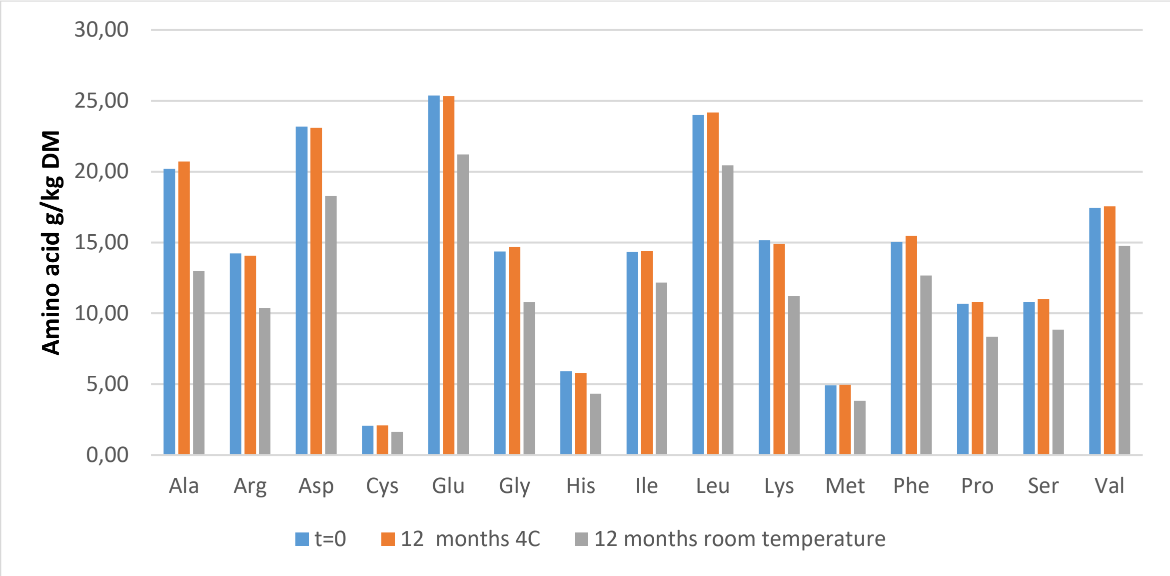

Figure 6. Amino acid composition of red clover protein paste at time of production and after 12 months of storage either at room temperature or at 4°C. Results g/ kg DM.

Fat and fatty acids

Red clover and perennial ryegrass have a high proportion of omega 3 fatty acids, constituted mainly of alpha-linolenic acid. During the biorefining process, the fat is concentrated in the protein paste resulting in a fat content of 10-14 % of the DM[2]. As this high content of fat, in particular unsaturated fat, may cause challenges with regard to shelf life, as unsaturated fat is prone to oxidative stess, it is highly relevant to analyse the fat content, the composition of the fatty acids and also the content of antioxidant during storage, as this is an indicator of the oxidative stress in the samples.

The content of fat in the paste from perennial ryegras sis shown in figure 7, whereas the content in red clover paste is seen in figure 8. The content of fat in samples from perennial ryegrass at time of production is 9.7 % of the sample DM. There is a small increase in the fat content expected to be caused by the degradation of other DM components.For red clover protein paste, the content of fat in the newly produced samples is approx 8.7-9.3 %, the difference between the two baseline samples demonstrating the variance during sampling. During storage, the content increases, indicating the degradation of other components, such as sugars. This degradation is more pronounced in red clover than in perennial ryegrass.

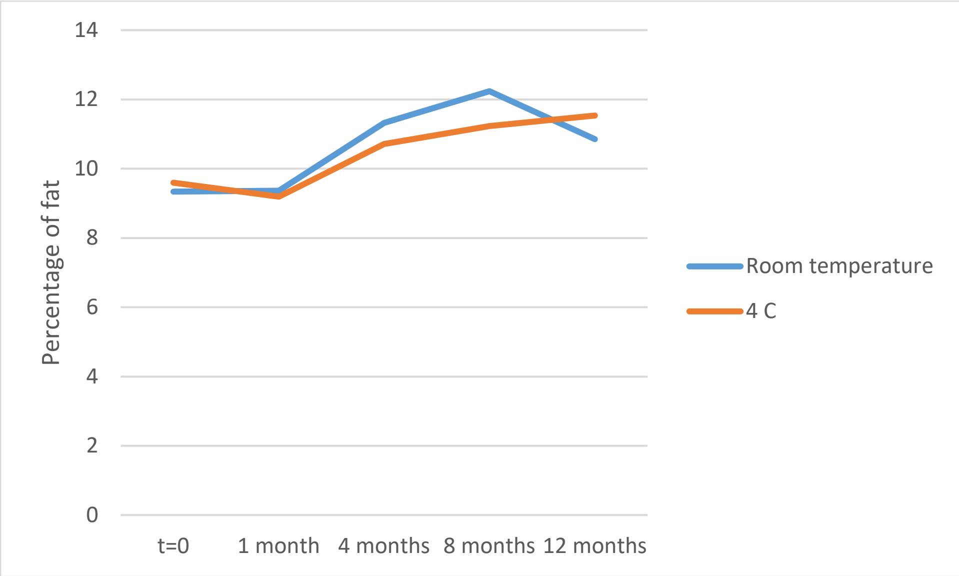

Figure 7 Fat content of perennial ryegrass protein paste at time of production and after 12 months of storage either at room temperature or at 4°C. Results g/ 100 g DM

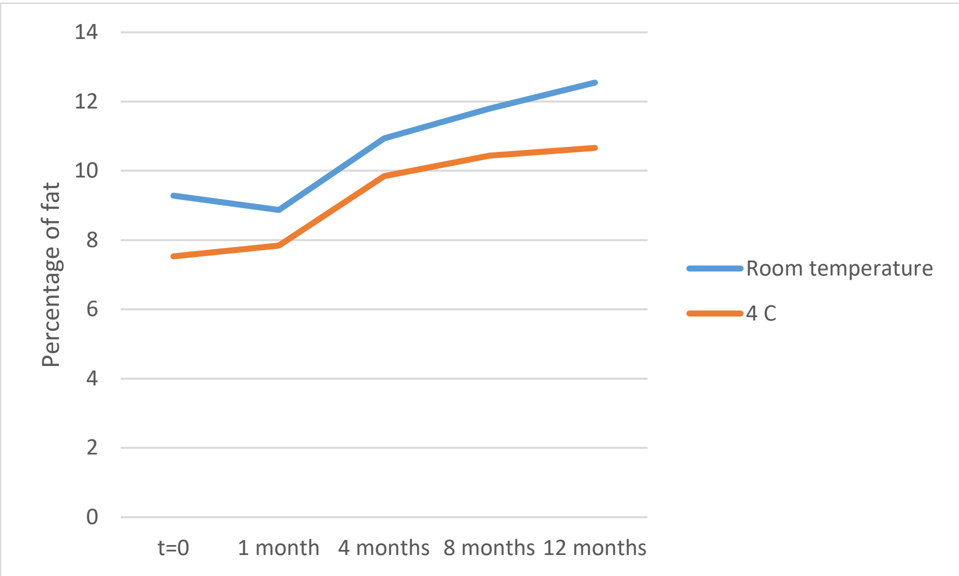

Figure 8 Fat content of red clover protein paste at time of production and after 12 months of storage either at room temperature or at 4°C. Results g/ 100 g DM

As the degree of fatty acid oxidation and hence also the shelf life of the protein paste is dependent on the level of unsaturation of the fatty acids, the distribution between saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in the samples and the effect of storage were analysed. The distribution in perennial ryegrass protein paste stored at room temperature is shown in figure 9, whereas samples stored at 4 degrees are shown in figure 10. All data are presented as % of DM.

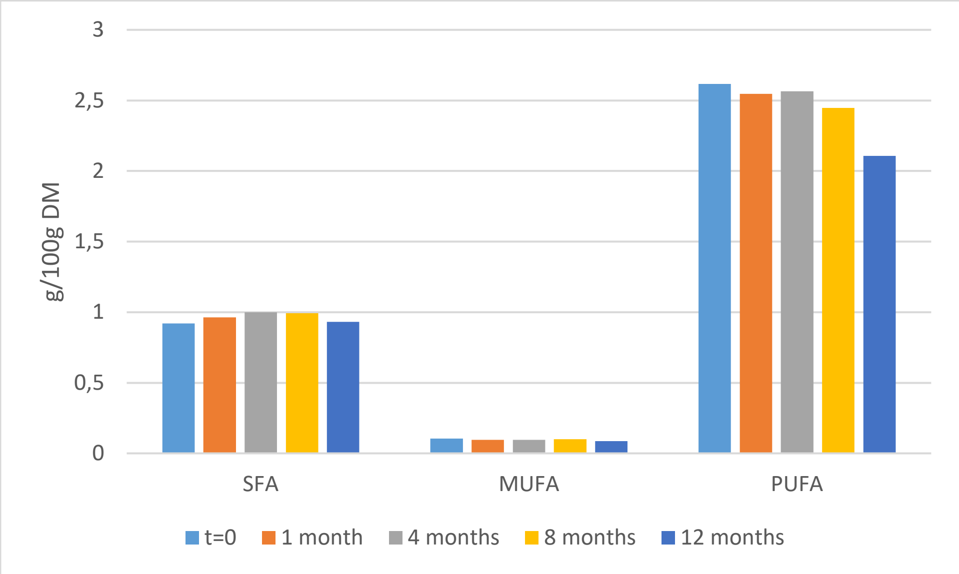

Figure 9 Content of saturated fat (SFA), monounsaturated fat (MUFA) and polyunsaturated fat (PUFA) in protein paste from perennial ryegrass stored at room temperature. Results are % af DM.

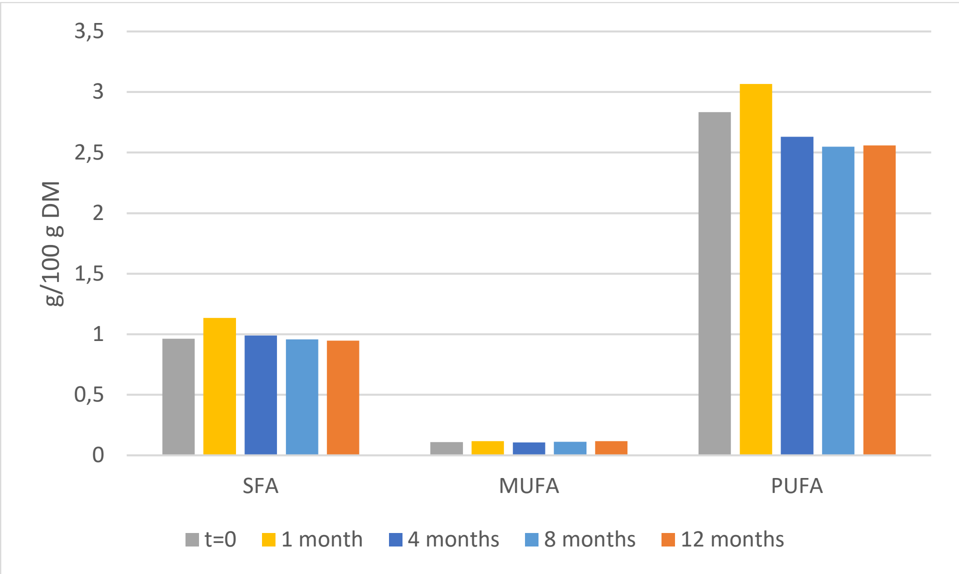

Figure 10 Content of saturated fat (SFA), monounsaturated fat (MUFA) and polyunsaturated fat (PUFA) in protein paste from perennial ryegrass stored at 4 degrees. Results are % af DM.

For perennial ryegrass, the content of SFA remained contant during storage, whereas the content of PUFA decreased. At storage at 4 degrees, the decrease was less pronounced, indicating that the low temperature can inhibit some of the degradation of PUFAs. The decrease was primarily caused by a decrease in the content of alpha-linolenic acid (data not shown), which is attenuated at low temperature storage.

For red clover, the distribution in protein paste stored at room temperature is shown in figure 11, whereas samples stored at 4 degrees are shown in figure 8. All data are presented as % of DM.

During storage at room temperature, there is a clear reduction in the content of PUFA, whereas the content of SFA and MUFA remains constant, similar to observations in perennial ryegrass. However, where low temperatures in perennial ryegrass attenuated this decrease, this is not the case with red clover paste, where samples stored at 4 degrees experience at clear decrease in PUFA, caused by a decrease in primarily alpha-linolenic acid (data not shown).

The difference in the effect of storage between the two species is expected to be caused by a very high content of endogenous oxidative enzymes in red clover. This will increase the oxidative stress in these samples and hence the degradation of PUFA.

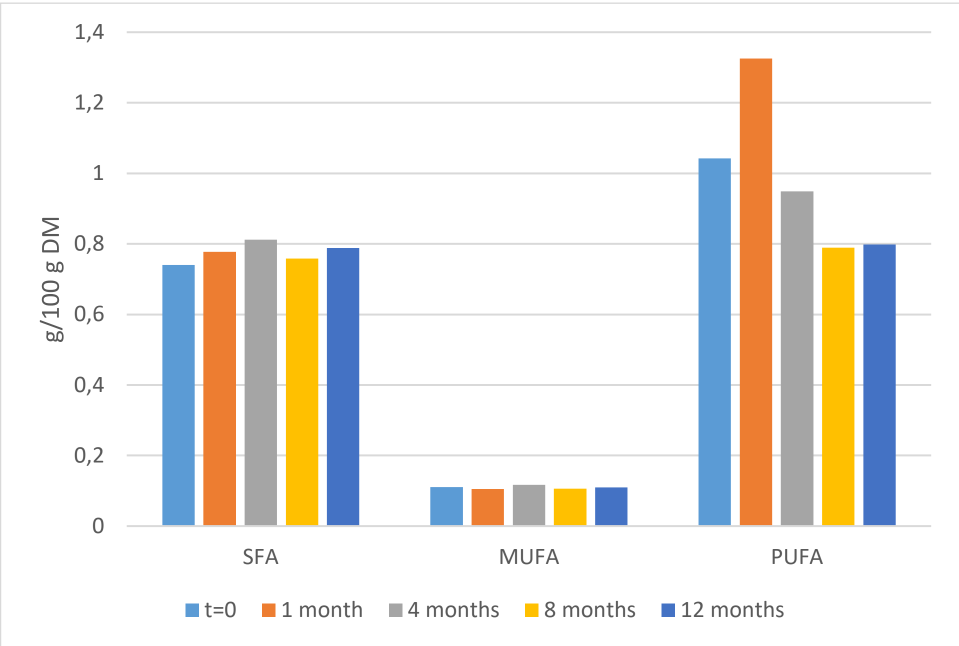

Figure 11 Content of saturated fat (SFA), monounsaturated fat (MUFA) and polyunsaturated fat (PUFA) in protein paste from red clover stored at room temperature. Results are % af DM

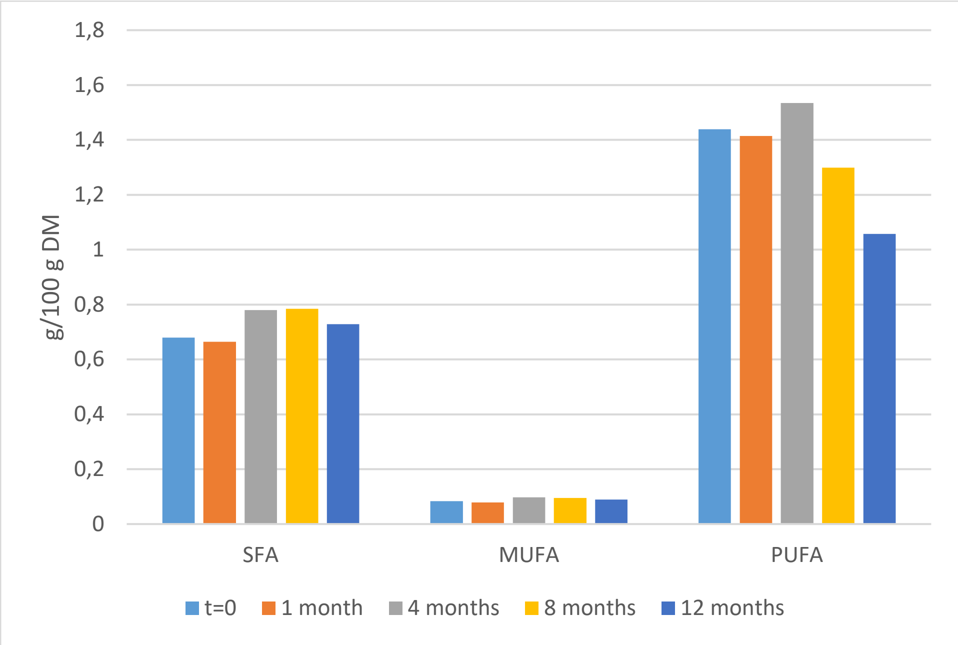

Figure 12 Content of saturated fat (SFA), monounsaturated fat (MUFA) and polyunsaturated fat (PUFA) in protein paste from red clover stored at 4 degrees. Results are % af DM

To further confirm a higher oxidative stress in red clover protein samples, the content of alpha-tocopherol, a form of vitamin E was analysed. In perennial ryegrass (figure 13) , the content remained stable at 4 degrees, whereas at samples stored at room temperature, a slight decrease was seen, indicating oxidative stress and hence the need for antioxidant action. This confirms the results from figure 9 and 10, that low temperature can reduce the oxidations in the perennial ryegrass samples and hence alpha-tocopherols are not needed. Figure 14 shows the content of alpha-tocopherol in protein paste from red clover. It is clear, that samples stored at room temperature had a reduced content of alpha-tocopherol demonstrating that this antioxidant is used in the process on reducing oxidative stress. At 4 degrees, we do not see the expected decrease in alpha-tocopherol content, but this may be cause by other antioxidant functioning to reduce the stress observed in figure 12.

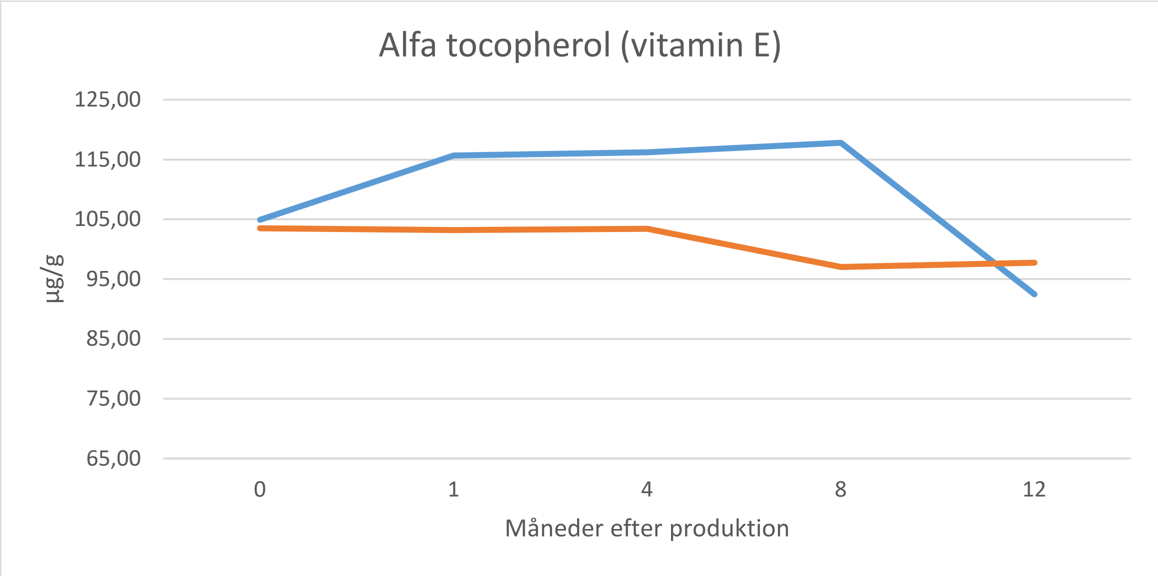

Figure 13 Content of Alpha-tocopherol in perennial ryegrass protein paste. Blue: room temperature. Red: 4 degrees

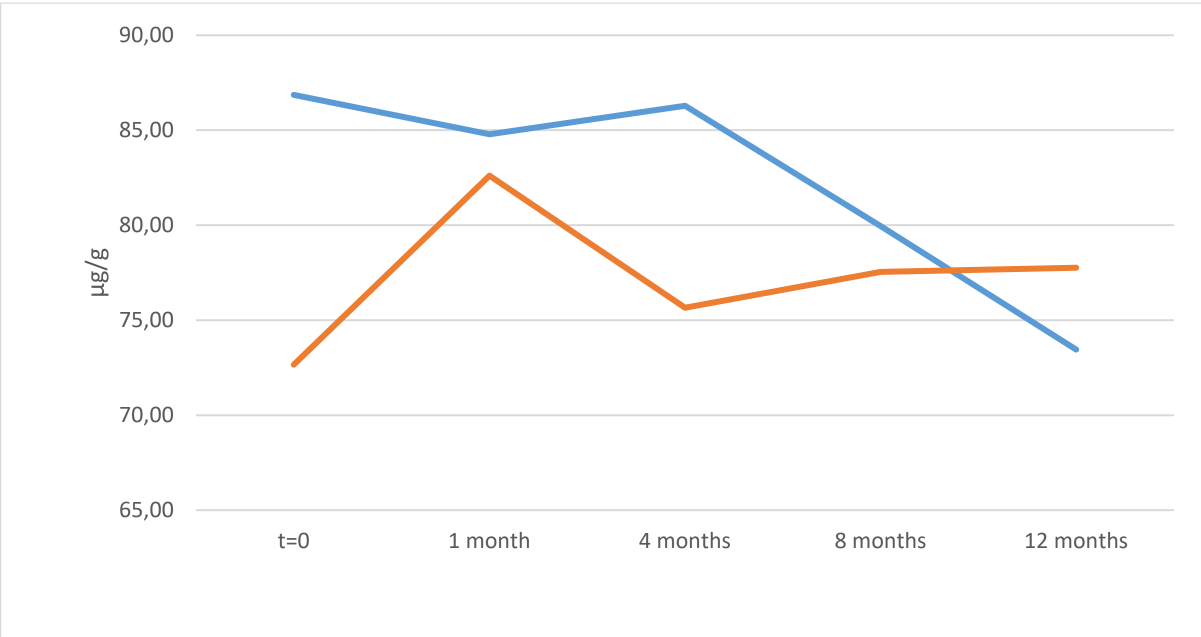

Figure 14 Content of Alpha-tocopherol in red clover protein paste. Blue: room temperature. Red: 4 degrees

<u>CONCLUSIONS:</u>

- No clear trend was observed in terms of pH, concentration of lactic acid and other organic acids or microbial growth during the storage of the wet protein pastes at room temperature or in the fridge during one-year storage. Consequently, it is difficult to predict the shelf life of the wet protein paste samples after storage under anaerobic conditions for a certain period.
- Changes in the pH and in the concentration of acids in the protein pastes during their storage indicate some microbial activity, especially after storage at room temperature, which might have compromised the quality of the wet protein pastes. Microbial activity in the wet protein pastes could be also related with protein degradation, especially under limited availability of C sources.
- The lactic acid concentration in the wet protein paste samples clearly determined their pH, due to the low pK_a of the lactic acid (3.86). Therefore, securing a relatively low pH (approx. 3.8) during the lactic acid fermentation of the green juice will probably result in protein pastes with a pH low enough so as to prevent microbial activity and thus, to ensure good preservation of the wet protein pastes.
- Large number of microorganisms were present in the wet protein pastes, probably resulting from the fresh plant material but also from soil contamination. Mostly, the amount of microorganisms in the protein pastes was significantly reduced with storage, especially at room temperature. However, the large microbial numbers found in the wet protein pastes could hinder their shelf life under anaerobic conditions for a later use of the protein pastes for feeding purposes. Consequently, sterilization of the wet protein pastes by means of mild heat treatment could be convenient in order to preserve the quality of the wet protein pastes upon storage.
- Under the conditions by which this experiment was conducted, it can be concluded that storage of wet protein paste at room temperature for 12 months reduces the content of crude protein and of the individual amino acids in red clover. Storage at 4 degrees stabilizes the protein content and composition.
- There is an oxidative pressure in the protein pastes, demonstrated by the reduction of polyunsaturated fatty acid content, which in perennial ryegrass can be attenuated by storage at 4 degrees. For red clover, the content of oxidative enzymes is very high, preventing the protective effect of low temperature. The results also demonstrate that increasing the storage will also increase the need for antioxidants, especially if samples are to be stored at room temperature. Therefore, if wet samples are to be stored long-term, it would be necessary to store at low temperatures to avoid protein loss and oxidative stress

1. Stødkilde L, Damborg VK, Jørgensen H, Lærke HN, Jensen SK: Digestibility of fractionated green biomass as protein source for monogastric animals. *animal* 2019:1-9.
2. Stødkilde L, Damborg VK, Jørgensen H, Lærke HN, Jensen SK: Nutritional evaluation of fractionated green biomass as protein source for monogastric animals. Submitted to Animal 2018; 2018.

3. Santamaría-Fernández M, Molinuevo-Salces B, Kiel P, Steenfeldt S, Uellendahl H, Lübeck M (2017) Lactic acid fermentation for refining proteins from green crops and obtaining a high quality feed for monogastric animals. J Clean Prod 162, 875-881.