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Microbial Profile of Gouda Cheese During Ripening in Two Independent Chambers – a Short Report

Piotr Kołakowski[1], Richard Podolak[2], Marta Kowalska[1]*

[1]Danisco Biolacta, Innovation, ul. Tuwima 1A, 10–747 Olsztyn, Poland
[2]Grocery Manufacturers Association, 1350 I Street NW, Suite 300, Washington, D.C. 20005, USA

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A study was undertaken to evaluate changes in microbial populations of nonstarter lactic acid bacteria (NSLAB), yeast and starter lactic acid bacteria (SLAB) in Gouda cheese in two independent chambers during the ripening process up to 12 weeks. No differences in populations of the tested group of microorganisms were observed at 4, 8 and 12 weeks in both the dairy and dairy-independent chambers. Populations of the analysed groups of LAB reached maximum numbers at week 4 of ripening and then gradually decreased with further aging, however with different dynamics for different species. The SLAB were the predominant microflora after salt treatment and accounted for 90% of the total microbial population in Gouda cheese. Cheese ripening led to the predomination of NSLAB and yeast populations and to a decrease in the population of SLAB. Homo- and heterofermentative vancomycin-tolerant *Lactobacillus spp.* constituted for the majority of the NSLAB populations. The yeast counts, at the initial populations of 4 $\log_{10}$ CFU/g, were increased by 2 logs after 4 weeks and were slightly reduced at 8 and 12 weeks of ripening. At 12 weeks of ripening, nonstarter *Lactobacillus spp*. enumerated at 25°C exceeded 90% of total LAB population while the yeast population comprised over 40% of the total LAB counts. The majority of NSLAB consisted of vancomycin-tolerant homo- and heterofermentative species of *Lactobacillus*.

INTRODUCTION

Cheese microflora consists mostly of two major groups which are starter lactic acid bacteria (SLAB) and secondary microorganisms. The strains of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris, Leuconostoc mesenteroides*, *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Lactobacillus delbruecki* are frequently used individually or in combinations as mesophilic and thermophilic bacterial starters in the production of cheese [Hoier *et al*., 2010]. The SLAB are responsible for acid production and are involved in the ripening process of cheese [Settanni & Moschetti, 2010]. The secondary microflora comprises of mainly nonstarter lactic acid bacteria (NSLAB) and yeasts. The NSLAB are usually unique to specific cheese varieties. The NSLAB generally do not grow well in milk [Cogan *et al*., 2007; Briggiler-Marco *et al*., 2007] and therefore do not contribute to acid production, however, they are able to grow during ripening of cheese [Agarwal *et al*., 2006; Veljovic *et al*., 2007; Kongo *et al*., 2009; Ciprovica & Mikelsone, 2011; Barakat *et al*., 2011; Mirzaei, 2011]. An increasing trend in the counts of NSLAB is typical in most cheeses. Several researches suggested that the NSLAB can contribute to the typical or atypical but desirable flavour development and to the organoleptic properties of particular cheeses. The NSLAB can also be responsible for off flavour development and overproduction of CO_2 in cheeses [Briggiler-Marco *et al*., 2007; Cogan *at al*., 2007; Randazzo *et al*., 2007; Martin-Platero *et al*., 2008; Kongo *et al*., 2009; Aljewicz *et al*., 2010; Bockelmann, 2010; Settanni & Moschetti, 2010]. Although widely variable, the majority of the NSLAB population consists of facultative or obligatory heterofermentative lactobacilli belonging to *Lb. casei*, *Lb. plantarum*, *Lb. paraplantarum*; *Lb. pentosus*, *Lb. brevis*, *Lb. curvatus*, *Lb. fermentum*, *Lb. buchneri*, *Lb. parabuchneri*, *Lb. coryniformis* and *Lb. rhamnosus* species [Svec *et al*., 2005; Cogan e*t al*., 2007; Randazzo *et al*., 2007; Veljovic *et al*., 2007; Zago *et al*., 2007; Gala *et al*., 2008; Martin-Platero et al., 2008; Rantsiou *et al*., 2008; Belletti *et al*., 2009; Colombo *et al*., 2009; Randazzo *et al*., 2010; Terzic-Vidojevic *et al*., 2009; Samelis *et al*., 2010; Ghotbi et al., 2011; Jokovic *et al*., 2011; Mlalazi *et al*., 2011; Morales *et al*., 2011]. Among LAB, species of pediococci and leuconostoc are also present but to a lesser extent [Cogan *at al*., 2007; Colombo *et al*., 2009; Randazzo *et al*., 2009; Colombo *et al*., 2010; Jokovic *et al*., 2011]. Populations of SLAB are high in number (about 10^8–10^9 CFU/g) at the beginning of the ripening process and gradually decrease during aging [Zago *et al*., 2007; Franciosi *et al*., 2008]. On the contrary, NSLAB population can increase by 4 – 5 log cycles within a few weeks of cheese ripening from very low counts in fresh curd (<10 CFU/g) [Briggiler-Marco *et al*., 2007; Cogan *at al*., 2007; Kongo *et al*., 2009]. The SLAB may exhibit different sensitivity and tolerance to antibiotics. Except *Leuconostoc,* LAB cheese starters belonging to *Lactococcus*, *Streptococcus* and homofermentative *Lactobacillus* genera are characterised

* Corresponding author: Tel.: +48 89 524 02 41; Fax: +48 89 524 02 03
E-mail: piotr.kolakowski@danisco.com (P. Kołakowski)

by very low tolerance to vancomycin below 10 μg/mL (MIC < 10). In contrast, most of NSLAB *spp*. demonstrate high tolerance to vancomycin (MIC > 400) [Kołakowski *et. al*., 2004; Orberg & Sandine, 1984; Simpson *et. al*., 1988]. Media used for SLAB enumeration supplemented with vancomycin are often used for determination and isolation of NSLAB from dairy products [Benkerroum *et al*., 1993; Cogan *et al*., 2007]. Secondary microflora such as yeasts are frequently found in many types of cheeses in the range of populations from 10^4 – 10^6 CFU/g to as high as 10^7 – 10^8 CFU/g. The presence of yeasts in cheese are not unusual due to favourable conditions for their development in cheese and their wide spreading in dairy environment [Wyder, 2001; Alessandria *et al*., 2010; Mirzael, 2011]. The significance of the presence of yeast depends on the particular type of cheese [Wyder, 2001; Colombo *et al*., 2009]. Yeasts can be key species in bacterial development in cheese but their influence on bacteria differs [Mounier *et al*., 2008]. In some cheeses, yeasts can cause defects such as excessive gas production, bitter taste, fruit flavours, increased acidity and changes in texture profile [Wyder, 2001; Bockelmann, 2010]. However, in many cases yeasts can impart unique characteristics to several cheeses due to their lipolytic and proteolytic activities, formation of aromatic compounds, and degradation of the lactic acid in the surface of certain cheeses [Wyder, 2001; De Freitas *et al*., 2009; Bockelmann, 2010].

It is commonly known that the presence of the nonstarter microflora in cheeses depends on the sanitary condition of processed milk, brine, and microbial biofilms formed on the surface of dairy equipments [Agarwal *et al*., 2006; Casey *et al*., 2006; Cogan *at al*., 2007; Franciosi *et al*., 2009; Terzic-Vidojevic *et al*., 2009; Colombo *et al*., 2010; Begovic *et al*., 2011].

To date, there is a little information regarding the effect of environmental conditions of the production area where process of cheese ripening takes place, on the development of nonstarter microflora. Thus, the objective of this study was to monitor NSLAB, yeasts and starter LAB population's changes in Gouda cheese during ripening in two independent chambers. This paper further examines and discusses the microflora composition of cheese *versus* starter bacterial microflora.

MATERIAL AND METHODS

Gouda cheese

Gouda cheese was produced at a modern Polish cheese factory in the region of Warmia and Mazuria. The cheese, after salting, was ripened up to 12 weeks at 12°C (± 1) in two independent chambers: the dairy industrial chamber (volume 1200 m^3) and the laboratory type chamber (volume 1.5 m^3). The parameters during the ripening process such as temperature and humidity were the same for both the industrial and laboratory chambers. Microbiological analyses were carried out just after salt treatment and at 4, 8 and 12 weeks of ripening.

Cheese culture

Starter cultures of *Lactococcus lactis* and *Leuconostoc mesenteroides* (Choozit TP03, Danisco A/S) species were used for the cheese production. Culture microflora were characterised by using the same plate methods as those used for the cheese microflora.

Microbiological analyses

The 500 g samples of cheeses were grounded mechanically and mixed. The cheese culture was used directly without pretreatment. A 10-g sample of cheese or starter culture was placed individually into sterile stomacher bags and diluted with 90 mL of sterile 2% (w/v) trisodium citrate solution (9 g/L). Samples were homogenized for 3 min in a Stomacher (BigMixer, Interscience, France) at its maximum speed. For enumeration of viable microorganisms, the samples were serially diluted in 0.1% peptone water and plated onto the following media: modified Nickels-Leesment (N-L) medium for total count of LAB and citrates ferment LAB [Nickels & Leesment, 1964]; Nickels-Leesment medium supplemented with 200 μg/mL of filter-sterilized vancomycin (Sigma) for NSLAB and citrates ferment NSLAB enumeration [Kołakowski *et al*., 2004]. Plates were incubated aerobically at 25°C and 42°C for 48 h. Colonies surrounded by a clear zone on N-L medium were considered to be citrate ferment positive. For total *Lactobacillus* and vancomycin-tolerant *Lactobacillus spp.* enumeration, the serially diluted samples were plated on deMan, Rogosa and Sharpe (MRS) medium at pH 5.4 and MRS medium supplemented with 200 μg/mL of vancomycin, respectively. Plates were anaerobically incubated at 25°C and 42°C for 72 h [IDF, 2003]. Selectivity of the methods used for enumeration of different LAB groups was confirmed by microscopic observations, biochemical tests including API (Biomerieux). However, due to high similarity between starter and non-starter leuconostocs, and mesophilic heterofermentative lactobacilli some overlapping should be still considered. Yeast counts were determined by plating the samples on yeast extract chloramphenicol agar and aerobic incubation at 25°C for 120 h as described by Rea *et al.* [1996].

The average count of duplicate plates from each sample was used to determine total viable cell counts. Counts were converted to $\log_{10}$ and recorded as CFU/g. The experiment was replicated two times.

RESULTS AND DISCUSSION

The microflora composition of cheese culture is presented in Table 1. Microflora of culture consisted of *Lactococcus lactis* and *Leuconostoc* species only. *Lactobacillus*, including homo- and heterofermentative species, was not found on the selected media (MRS adjusted to pH 5.4 at both temperatures 25 and 42°C, and N-L with vancomycin at 42°C) in the cheese culture at the detectable level of 0.1 g. The total population of LAB reached 11.4 logs CFU/g. Analyses of the approximate proportion of bacterial populations in cheese culture showed that homofermentative *Lactococcus lactis* was the predominating bacteria and constituted for 85% of the total number of bacterial counts. The strains of *Leuconostoc mesenteroides* and *Lactococcus lactis* subsp. *lactis* var. *diacetylactis*, both with the capacity to ferment citrates, reached 10.6 logs CFU/g and constituted 15% of total LAB counts enumerated on N-L medium at 25°C. The counts

TABLE 1. Microflora profile of Choozit TP03 at 25°C and 42°C.

Microflora	Incubation temperature	Count
	(°C)	(Log_{10} CFU/g)
Total LAB count	25	11.4
	42	< 1
Citrates ferment LAB	25	10.6
	42	< 1
Vancomycin-tolerant LAB	25	10.0
	42	< 1
Vancomycin-tolerant citrates ferment LAB	25	10.0
	42	Nd[1]
Lactobacillus spp.	25	Nd[1]
	42	Nd[1]
Yeast	25	Nd[1]

[1] Nd – non detected at the level of 0.1 g.

of *Leuconostoc mesenteroides* species that were enumerated at the same medium but supplemented with 200 μg/mL of vancomycin, did not exceed 5% (10 logs CFU/g). All colonies enumerated on the N-L medium with vancomycin showed the ability to ferment citrates and belonged to *Leuconostoc* species. The high vancomycin tolerance of *Leuconostoc spp*. and low vancomycin tolerance of *Lactococcus lactis spp*. used in dairy industry were reported in many studies [Kołakowski *et. al*., 2004; Svensson, 1994; Orberg & Sandine, 1984]. The increase of incubation temperature of N-L medium from 25°C to 42°C resulted in a negligible level (< 10 CFU/g) of citrates ferment and vancomycin tolerant LAB in cheese culture and additionally confirmed that colonies enumerated on N-L medium with vancomycin belonged to *Leuconostoc* species.

The microflora composition of Gouda cheese during ripening is presented in Table 2. NSLAB species in Gouda cheese were enumerated on the following media: MRS medium adjusted to pH 5.4 at both 25 and 42°C (meso- and thermophilic *Lactobacillus*); N-L medium with vancomycin at 42°C (thermophilic vancomycin-tolerant homofermentative *Lactobacillus*), and N-L medium with vancomycin at 25°C (mesophilic homofermentative *Lactobacillus*). Citrates ferment LAB species enumerated on N-L with vancomycin at 25°C can belong to starter and nonstarter *Leuconostoc* spp. and nonstarter heterofermentative *Lactobacillus* spp.

The population of total mesophilic LAB in salted cheese was 6.9 logs CFU/g while that of thermophilic ones reached 1.8 logs CFU/g and increased to 7.4 and 6.7 logs CFU/g after

TABLE 2. Microflora profile of Gouda cheese during ripening in two independent chambers.

Microflora	Incubation temperature	Ripening chamber [1, 2]	Ripening time			
			Weeks			
			After salting	4	8	12
	(°C)		(Log_{10} CFU/g)			
Total LAB count	25	1	6.9	7.4	7.1	5.7
	42		1.8	6.7	6.7	4.7
	25	2	6.9	7.5	7.1	5.6
	42		1.8	6.6	6.6	4.8
Citrates ferment LAB	25	1	6.1	6.6	6.5	5.0
	42		Nd[1]	Nd[1]	Nd[1]	Nd[1]
	25	2	6.1	7.3	6.0	5.2
	42		Nd[1]	Nd[1]	Nd[1]	Nd[1]
Vancomycin-tolerant LAB	25	1	5.7	7.0	6.9	5.6
	42		Nd[1]	6.5	6.1	4.6
	25	2	5.7	7.1	6.9	5.5
	42		Nd[1]	6.5	6.0	4.6
Vancomycin-tolerant citrates ferment LAB	25	1	5.5	6.3	6.2	4.57
	42		Nd[1]	Nd[1]	Nd[1]	Nd[1]
	25	2	5.5	6.3	6.0	4.6
	42		Nd[1]	Nd[1]	Nd[1]	Nd[1]
Lactobacillus spp.	25	1	3.7	6.9	6.9	5.7
	42		1.2	6.3	6.1	4.5
	25	2	3.7	7.0	6.5	5.6
	42		1.2	6.3	5.8	4.4
Yeast	25	1	4.1	6.6	6.6	5.3
		2	4.2	6.5	6.4	5.2

Nd[1] – non detected at the level of.0.1 g; 1 – dairy chamber; 2 – dairy-independent chamber. Results are mean values of two replicated experiments.

4 weeks of aging, respectively. Starter lactic acid bacteria were the major component of the microflora of Gouda cheese after salting, including a similar ratio for citrates ferment and vancomycin-tolerant mesophilic bacteria (17% and 5% of total mesophilic LAB count, respectively). Mesophilic population of vancomycin-tolerant LAB in salted cheese accounted for 5.7 logs CFU/g and vancomycin-tolerant LAB, with the ability to ferment citrates, for 5.5 logs CFU/g. It means that approximately 25% of vancomycin-tolerant lactic acid bacteria enumerated on N-L medium at 25°C were not of cheese culture origin and did not belong to *Leuconostoc mesenteroides* due to the lack of clear zones around bacterial colonies. Also, contrary to the cheese culture composition, *Lactobacillus spp*. in salted Gouda were detected at both 25°C and 42°C reaching 3.7 and 1.2 logs CFU/g. Unexpectedly, the high number of yeasts exceeding 4 logs CFU/g in Gouda cheese after salting was counted. This is contrary to the study conducted by Welthagen & Viljoen [1998] who reported 2 logs of yeast populations after one-day ripening of Gouda cheese. Although, yeast counts may vary between dairy plants and even between subsequent days in the same plant according to Viljoen & Greyling [1995] and Welthagen & Viljoen [1998], the brine used for salting was mainly responsible for the high rate of yeast contamination in Gouda cheese.

No differences were observed in the viable numbers of lactic acid bacteria and yeast counts composition at 4-, 8- and 12 weeks of ripening in dairy and dairy-independent chamber (Table 2). A 4-week aged cheese was characterised by the highest population of the total LAB count, NSLAB and yeasts. In a study carried out by Welthagen & Viljoen [1998], a steadily increasing number of yeasts and LAB were observed during Gouda cheese ripening over a period of 32 days and 18 days, respectively. In another study, the researchers reported that the total counts of LAB in Cheddar cheese after the initial increase started to diminish after 14 days of ripening [Agarwal *et al*., 2006]. In our studies, after 4 weeks of ripening, the population of yeasts and all groups of lactic acid bacteria substantially increased with nonstarter microflora showing the highest growth tendency. The total counts of thermophilic and mesophilic *Lactobacillus*, mesophilic vancomycin-tolerant LAB and yeasts increased by 5, 3.2, 1.5 and 2 log, respectively while total counts (mainly starter) of mesophilic LAB increased only by 0.5 log cycle. Furthermore, the ripening process resulted in a steadily reduction of bacterial and yeast counts, but at different rates for different groups of microflora. Generally, the bacterial populations for tested groups were reduced maximally by 0.5 logs at 8 week of ripening. At 12-week aging, the populations of total mesophilic LAB declined almost by another 1.5 log cycles and the NSLAB and yeast became the most dominant viable microorganisms. The contribution of yeasts was 6–12%, 22–30% and 41–44% of total count of LAB in 4-, 8- and 12-week aged Gouda cheeses, respectively.

Mesophilic vancomycin-tolerant LAB constituted for more than 40% of total mesophilic LAB in 4-week aged cheese and more than 60 and 80% in 8- week and 12-week aged cheeses, respectively. The populations of *Lactobacillus spp*. enumerated on MRS medium at pH 5.4 and 42°C (Table 2) increased in counts from 10^1 CFU/g) in fresh cheese to 6 logs CFU/g after four weeks of ripening. This is in agreement with the results of Casey *et al*. [2006] and Zago *et al*. [2007], who reported that the NSLAB in cheese were mostly predominated by *Lactobacillus* and less by *Leuconostoc spp.* and other nonstarter lactic acid bacteria genera.

The temperature of 12°C in a cheese ripening chamber did not favour the growth of thermophilic bacteria. More than 80% of *Lactobacillus spp*. enumerated on MRS medium pH 5.4 at temperature 25 and 42°C were vancomycin-tolerant regardless of the time of ripening (data not shown). Therefore, it should be concluded that high vancomycin tolerance heterofermentative *Lactobacillus spp*. were the predominant microflora in Gouda cheese mostly due to their wide optimum temperature of growth [Settani & Moschetti, 2010].

CONCLUSIONS

This study was designed to evaluate NSLAB, yeasts and starter LAB population's changes in Gouda cheese during ripening in two independent chambers. Maturation place did not effect Gouda cheese microflora. No differences were observed in microflora composition among 4-, 8- and 12--week aged cheeses ripened in dairy and dairy-independent chamber. Cheese ripening led to intensive increase of microbial population up to 4 weeks and substantially decreased up to 12 weeks. *Lactobacillus spp*. presented at a very low initial level increased by 1.5–6 log in salted Gouda cheese after four weeks of ripening. On the contrary, the total LAB count increased by 0.5 log cycle. At the same time, yeast count increased from 4 logs to 6 logs CFU/g. After 12 weeks of ripening, the total viable mesophilic LAB counts declined to approximately 2% of their maximum numbers achieved in the 4 week ageing cheese. Nonstarter *Lactobacillus spp*. and yeasts were the dominant microflora in ripened cheese. The majority of *Lactobacillus* population consisted of homo- and heterofermentative vancomycin-tolerant species growing well in a wide range of temperatures. In 12-week Gouda cheese, *Lactobacillus spp*. enumerated at 25°C exceeded 90% and yeasts over 40% of the total mesophilic count of LAB.

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