Survival of pathogens in "Graviera Kritis" cheese made with raw and pasteurized milk

Survival of pathogens in “Graviera Kritis” cheese made with raw and pasteurized milk

Samouris G., DVM, PhD, Zdragas A., DVM, MSc, PhD, Vafeas G., TE, Belibasaki S., DVM, PhD, DipECAR.

Veterinary Research Institute of Thessaloniki, National Agricultural Research Foundation (N.AG.RE.F.), Thessaloniki, Greece

ABSTRACT. A field trial was carried out to investigate the hygienic status of Graviera 'Kritis' cheese made with raw and pasteurized milk. Graviera was manufactured in a small plant in Crete under traditional manufacturing conditions. Forty-two samples were examined during the manufacturing process for Coliforms, Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, Salmonella spp., Escherichia coli O157:H7 and VRE (Vancomycin Resistant Enterococci). All samples were negative for Salmonella spp., E. coli O157:H7 and VRE. Populations of Coliforms and E. coli before cooking of the curd were 3-3.5 log_{10} MPN/g higher in cheese made with raw milk in comparison to that made with pasteurized milk and populations of both bacteria decreased sharply during the aging period in both types of cheese. L. monocytogenes type 4b was isolated from the cheeses of two trials until after the salting period and remained under the detection limit of 100 cfu/g. At the end of the ripening period, all the examined bacteria were declined or eliminated to safe level.

Keywords: Graviera cheese, raw milk, pathogen, safety

ΠΕΡΙΑΨΗ. Για τη μελέτη της υγιεινής κατάστασης της «Γραβιέρας Κρήτης» έγιναν σε ένα παραδοσιακό τυροκομείο της Κρήτης, με τον παραδοσιακό τρόπο, πειραματικές τυροκομήσεις από νωπό και παστεριωμένο γάλα. Εξετάσθηκαν 42 δείγματα από τα διάφορα στάδια της διαδικασίας παραγωγής για την παρουσία Coliforms, Escherichia coli, Staphylococcus aureus Listeria monocytogenes, Salmonella spp., Escherichia coli O157:H7 και VRE (Vancomycin Resistant Enterococci). Όλα τα δείγματα ήταν αρνητικά για την παρουσία Salmonella spp, Escherichia coli O157:H7 και VRE. Οι πληθυσμοί των Coliforms και της E. coli πριν από το στάδιο της αναθέρμανσης της τυρομάζας, ήταν 3-3.5 log_{10} MPN/g υψηλότεροι στα τυριά που παρασκευάστηκαν με νωπό γάλα σε σχέση με εκείνα που παρασκευάστηκαν από παστεριωμένο γάλα. Οι πληθυσμοί και των δύο προηγούμενων βακτηρίων μειώθηκαν απότομα κατά τη διάρκεια της περιόδου ορίμανσης του τυριού και στους δύο τελευταίους τυριών. Η Listeria monocytogenes, τύπος 4b αποκαλύφθηκε στα τυρία δύο πειραματικών τυροκομήσεων και επιβίωσε σε αυτά μέχρι το στάδιο παραγωγής μετά την αλάτιση. Στα τυρία αυτά μετά το στάδιο της αλάτισης όπως και σε όλα τα υπόλοιπα παρέμεινε σε μη ανιχνεύσιμους αριθμούς. Στο τέλος της περιόδου ορίμανσης όλα τα βακτήρια που εξανέσθησαν είχαν μειωθεί σε σημαντική μεγεθούς για την κατανάλωση επιθέματα.

Λέξεις ευρετηρίασης: Γραβιέρα Κρήτης, νωπό γάλα, παθογόνα βακτηρία

Correspondence: Samouris G.
P.O.B. 60272, 570 01, Thermi-Thessaloniki, Greece
Tel.: +30 2310 365382, Fax: +30 2310 365371, e-mail:Dfoodtox@otenet.gr

Submission date: 06.05.2011
Approval date: 14.09.2011

Περιεχόμενα: Αναλύσεις της κατάστασης της «Γραβιέρας Κρήτης» στην παραδοσιακή τυροκομία. Η μελέτη του συνόλου των παθογόνων βακτηρίων που μπορεί να παρασκευάζονται από νωπό και παστεριωμένο γάλα. Εξετάσθηκαν 42 δείγματα από τα διάφορα στάδια της διαδικασίας παραγωγής για την παρουσία Coliforms, Escherichia coli, Staphylococcus aureus Listeria monocytogenes, Salmonella spp., Escherichia coli O157:H7 και VRE (Vancomycin Resistant Enterococci). Όλα τα δείγματα ήταν αρνητικά για την παρουσία Salmonella spp, Escherichia coli O157:H7 και VRE. Οι πληθυσμοί των Coliforms και της E. coli πριν από το στάδιο της αναθέρμανσης της τυρομάζας, ήταν 3-3.5 log_{10} MPN/g υψηλότεροι στα τυρία που παρασκευάστηκαν με νωπό γάλα σε σχέση με εκείνα που παρασκευάστηκαν από παστεριωμένο γάλα. Οι πληθυσμοί και των δύο προηγούμενων βακτηρίων μειώθηκαν απότομα κατά τη διάρκεια της περιόδου ωρίμανσης του τυριού και στους δύο τελευταίους τυριών. Η Listeria monocytogenes, τύπος 4b αποκαλύφθηκε στα τυρία δύο πειραματικών τυροκομήσεων και επιβίωσε σε αυτά μέχρι το στάδιο παραγωγής μετά την αλάτιση. Στα τυρία αυτά μετά το στάδιο της αλάτισης όπως και σε όλα τα υπόλοιπα παρέμεινε σε μη ανιχνεύσιμους αριθμούς. Στο τέλος της περιόδου ορίμανσης όλα τα βακτήρια που εξανέσθησαν είχαν μειωθεί σε σημαντική μεγεθούς για την κατανάλωση επιθέματα.

Άξονες ευρετηρίασης: Γραβιέρα Κρήτης, νωπό γάλα, παθογόνα βακτηρία
Cheeses have been made with raw milk all over the world for many generations. Many countries produce cheeses with raw milk, as does Greece, where a lot of traditional raw milk cheeses are made. Islands and mountainous areas continue to preserve the Greek tradition of making cheese with raw milk. ‘Graviera’ is one of the hard Greek cheeses which were traded and distinguished by the name of the region where they are produced like Crete, Naxos, Tinos and Lamia. Graviera Kritis, along with Graviera Naxos and Agrafon, are PDO Greek cheeses. “Graviera Kritis” is exclusively produced in Crete from ewe’s milk or mixtures of ewe’s milk with small quantities of goat’s milk. “Graviera Kritis” is a high quality hard cheese produced with the aid of both propionic and lactic acid bacteria fermentation, which has a slightly sweet taste and very pleasant organoleptic properties. It contains a moisture up to 35-38%, fat in dry matter at least 40-47% and no more than 2% salt (Moatsou et al. 2004).

Traditionally, it has been assumed that harmful microorganisms in cheese made with raw milk may be eliminated or reduced to safe levels during the cheese making process. Pathogenic microorganisms in raw milk die off during the cheese making, due to high acidity and competition with starter cultures (Fox 1993). Ripening acts as a natural selector during which lactic acid bacteria inhibit pathogens through the production of several antimicrobial compounds, such organic acids, hydrogen peroxide and bacteriocins (Topisirovic et al. 2006, Caridi et al. 2003). However, the risk of the primary pathogens {Salmonella spp., L. monocytogenes, E. coli O157:H7, VRE and Staph. aureus} (Bachmann and Spahr 1995) required to curdle the milk within 25-30 minutes was added. The raw milk was warmed at 35 °C and the rennet was added. Then, the same steps were followed for the cheese production with raw and pasteurized milk. The curd was cut into small pieces (the size of a corn) and it was then stirred for 10 min and cooked at 48-50°C with continuous stirring. The curd is usually cooked by slow cooking (1°C/2½ min) until a temperature of 43-44°C is reached and then by fast cooking (1°C/1-1½ min) until a temperature of 50°C is reached. Stirring continued.
thereafter for another 20 min and then the curd was left to settle at the bottom of the vat. After removing the greatest part of the whey, the curd was cut into pieces, each one large enough to fill one mould. Each mould was lined with a cheese cloth. The cheese in the moulds was pressed for 24h by low pressure (no more than twice the weight of the cheese), initially, which was gradually increased to 12 times the weight of the cheese. The cheese cloth was changed three times. After pressing, the cheese was put into brine (20% salt) for two days and it was then transferred to a ripening room (with a temperature of 14-17 °C) for two months. It was turned upside down every morning for the first 15 days and then every week. Then, the cheese was kept at a lower temperature (<6 °C) again to continue ripening. During ripening, the cheese was surface-salted by repeating a dry salting process 20-30 times. The total ripening time was three months.

Sampling for microbiological examination was done on the curd (day 0), before cooking (1-2h), before salting (1st day), after salting (3rd day), during the ripening period (60th and 90th days) and during storage (120th day). A microbiological examination was conducted in triplicate samples per time per type of cheese to estimate the hygienic status of the cheese in the production line.

Microbiological analysis

The microbiological analysis was conducted using ISO-based protocols. Overall, 42 samples were examined to enumerate Coliforms (IDF 1998), *E. coli* (ISO16649-3), *Staphylococcus aureus* (6888-2). The presence and enumeration of *Listeria monocytogenes* was carried out according to ISO 11290-1 11290-2, while *Salmonella* spp. and *E. coli* O157:H7 was based on ISO 6579:2002/DAM and 16654/01, respectively.

Vancomycin Resistant Enterococci (VRE) were detected using kanamycin azide agar (Merck KGaA, Germany) supplemented with 10mg/L vancomycin for the primary VRE detection. Suspected colonies were subjected to biochemical tests for genus confirmation (NaCl 6.5%, pH 9.6, 45 °C), followed by carbohydrate fermentation for species confirmation. All certain VRE species, if present, were tested for sensitivity in Vancomycin with Minimal Inhibition Concentration (NCCLS 2006).

Chemical analysis

Three parameters were determined in this survey: moisture, established by drying to a constant weight at 105 °C; pH, determined electrometrically; and the NaCl in the moisture of the cheese, which was estimated as \( \frac{(NaCl \text{ percentage} \times 100)}{\text{moisture + NaCl percentage}} \).

### Statistical Treatment of Data

Analyses of variance were performed on data obtained at different stages of ripening after log transformation for microbial counts, using the MINITAB 15.0 statistical software.

### RESULTS

The microbiological analysis of “Graviera” samples produced with pasteurized and raw milk revealed the absence of *Salmonella* spp., *E. coli* O157:H7 and VRE (*Vancomycin Resistant Enterococci*).

At time 0, the population of *coliforms* (Table 1) was \( 5.07 \pm 0.15 \) and \( 1.64 \pm 0.18 \) log MPN/g in the curd produced with raw and pasteurized milk, respectively. At the end of the third day (after the salting process), the population of coliforms in cheese made with pasteurized milk increased, reaching the numbers observed in the curd prepared with raw milk. During the ripening period, coliforms started to decline gradually in both trials, resulting in a sharply decreased count on the 120th day of storage. At this point, the counts in the raw and pasteurized milk were not statistically significant (1.01 ±0.41 and 1.10±0.63 log MPN/g, respectively). The *Escherichia coli* populations (Table 1) at time 0 were 3.72±0.75 and 0.70±0.23 log MPN/g in cheese prepared with raw and pasteurized milk, respectively. The population of *E. coli* in pasteurized reheated curd increased after the salting period. *E. coli* populations started to decline after the ripening period, resulting in a sharp decrease at the 120th day of storage, where the counted numbers were \( 0.85 \pm 0.39 \) and \( 0.46 \pm 0.00 \) log MPN/g for raw and pasteurized milk cheese, respectively.

Coagulase Positive Staphylococci (CoPS) were counted at the time 0 and had values of 4.08±0.20 and 1.19±0.39 cfu/g for the raw milk and curd made with pasteurized, respectively. At the end of the third day (after the salting process), the population of CoPS in the raw reheated curd had increased, while the population of CoPS had been reduced significantly during the ripening period in all samples and it was not
Table 1. Changes in coliforms, E. coli and CoPS1 populations during the processing of Graviera cheese prepared with raw (R) and pasteurized (P) milk (log MPN/g)

<table>
<thead>
<tr>
<th>Production stage</th>
<th>Day</th>
<th>Coliforms (log MPN/g)</th>
<th>E. coli (log MPN/g)</th>
<th>S. aureus (log cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>P</td>
<td>R</td>
</tr>
<tr>
<td>Before cooking</td>
<td>0</td>
<td>5.07±0.15</td>
<td>1.64±0.18</td>
<td>3.72±0.75</td>
</tr>
<tr>
<td></td>
<td>2h</td>
<td>4.62±0.51</td>
<td>1.90±0.29</td>
<td>3.21±0.58</td>
</tr>
<tr>
<td>After cooking</td>
<td>1</td>
<td>5.38±1.00</td>
<td>4.71±0.66</td>
<td>5.38±1.00</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.50±0.72</td>
<td>5.24±0.50</td>
<td>4.72±0.88</td>
</tr>
<tr>
<td>After salting</td>
<td>60</td>
<td>3.87±0.50</td>
<td>5.24±0.72</td>
<td>3.87±0.50</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>2.90±0.23</td>
<td>3.93±0.33</td>
<td>2.90±0.23</td>
</tr>
<tr>
<td>Ripening</td>
<td>120</td>
<td>1.10±0.63</td>
<td>1.01±0.41</td>
<td>0.46±0.00</td>
</tr>
</tbody>
</table>

Means within the same characteristics with different superscripts that differ significantly (P<0.05)

Values represent mean ± SE of the three trials

Table 2. Values1 of pH, moisture and NaCl content of “Graviera Kritis” cheese made from raw (R) and pasteurized (P) ewes' milk.

<table>
<thead>
<tr>
<th>Cheese type</th>
<th>Days</th>
<th>Time</th>
<th>Before cooking</th>
<th>After cooking</th>
<th>Before salting (1 day)</th>
<th>After salting (4 days)</th>
<th>45</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td></td>
<td>R</td>
<td>6.27±0.23</td>
<td>6.48±0.15</td>
<td>5.34±0.049</td>
<td>5.38±0.075</td>
<td>5.38±0.2</td>
<td>5.07±0.079</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>6.76±0.04</td>
<td>6.77±0.02</td>
<td>5.97±0.16</td>
<td>5.94±0.11</td>
<td>5.52±0.29</td>
<td>5.11±0.23</td>
</tr>
<tr>
<td>Moisture %</td>
<td></td>
<td>R</td>
<td>67.1±4.15</td>
<td>53.69±9.48</td>
<td>45.51±3.008</td>
<td>41.92±4.14</td>
<td>39.44±1.1</td>
<td>33.6±1.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>67.42±5.83</td>
<td>53.56±5.41</td>
<td>47.24±3.83</td>
<td>42.92±3.37</td>
<td>36.85±8.45</td>
<td>31.52±3.46</td>
</tr>
<tr>
<td>NaCl-in-moisture %</td>
<td></td>
<td>R</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3.05±0.82</td>
<td>2.53±0.28</td>
<td>2.417±0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3.41±0.688</td>
<td>3.37±0.538</td>
<td>3.128±0.209</td>
</tr>
</tbody>
</table>

Means within the same characteristic with different superscripts that differ significantly (P<0.05)

1 Mean of the three batches of cheese
2 Not determined

Listeria monocytogenes was isolated from the first and second trial on the first day of cheese making, before cooking in raw milk cheeses, but the pathogen was below the 100cfu/g in all of the remaining samples. Enumeration of Listeria monocytogenes revealed numbers of 180cfu/g for the first positive sample and 220cfu/g for the second sample. Serotyping of these isolates showed the presence of the 4b type.

Raw milk cheeses’ pH before salting (1 day) and at the end of the ripening period (90 days) was 5.30±0.049 and 5.07±0.079, respectively, while in pasteurized milk cheeses it was 5.97±0.16 and 5.11±0.23, respectively. The moisture (%) and the content of NaCl in moisture (%) at the end of the ripening period were 33.6±1.96 and 2.417±0.14, respectively, for the cheese made with raw milk and 31.52±3.46 and 3.128±0.209, respectively, for the cheese made with pasteurized milk (Table 2).
DISCUSSION

Coliforms and E. coli are indicators of poor hygiene and their presence in food is indicative of possible fecal or environmental contamination during processing. The populations of Coliforms and E. coli in cheese made with raw milk were 3-3.5 log_{10} MPN/g higher than those in cheese made with pasteurized milk due to inappropriate conditions of milking, storage and cheese manufacturing. Increasing numbers of Coliforms and E. coli in cheese samples made with pasteurized milk were observed until the salting period and this was attributed to the poor hygiene conditions, the abidance of curd in ambient temperature and pH level, which ranged from 6.76 before cooking to 5.9 after the salting period. On the other hand, decreasing of indicators in raw milk cheese is due to the role of lactic acid bacteria as antagonistic factor (Lindgren et al. 2006) and the higher pH dropping ranging from 6.27 to 5.3 in the same time period. Counts of Coliforms and E. coli during the ripening and storage periods decreased significantly (P< 0.05) in both cheese types. Similar results were, also, found in hard Austrian Bergkaese cheese (Eliskases-Lechner et al.1999) and in traditional feta cheese (Manolopoulou et al. 2003). It is worth noting that the decline of bacterial indicators in raw milk cheese is not statistically significant as that in the product produced by pasteurized milk and the elimination of the indicators in both cheese types meets the EC Recommendations 2073/2005 for safety.

The primary pathogens posing a risk to cheese safety include Listeria monocytogenes, Salmonella spp., E. coli O157: H7, Staphylococcus aureus and VRE. The manufacturing process in hard cheeses, like graviera, is not favourable for growing pathogenic bacteria because of low moisture, low pH and long ripening period. (Cordano and Rocourt 2001).

Listeria monocytogenes is considered to be a widespread environmental contaminant detected in cheese plants. It has been shown (Brito 2007) that the environment and sanitation of the processing plant, as well as the post-processing contamination, play an important role in the prevention of Listeria monocytogenes. Decrease of Listeria monocytogenes, below the level of 100cfu/g at the 4th day after salting in the present study, is attributed to the drop in the pH (from 6.27 to 5.3) in combination with the decreasing of moisture from 67.1% to 41.92% of cheese. Other studies showed that neither Listeria monocytogenes nor enterotoxigenic Staphylococcus aureus could grow in the cheese core during ripening and on the cheese surface post-ripening (Samelis et al. 2009b, Giannou et al. 2009), but survival and not inactivation of Listeria monocytogenes at levels below 100cfu/g occur in the core of traditional Greek Graviera cheese during fermentation, ripening and storage (Samelis et al. 2009a). The antilisterial effect of Lactic Acid Bacteria (LAB) has been documented by Rodriguez et al. (2000) in hard cheeses from raw milk, as well as in Graviera from raw and thermized milk (Samelis et al. 2009b, Giannou et al. 2009, Samelis et al. 2010). Furthermore, the bacteriocin nisin produced by certain strains of Lactococcus lactis subsp lactis, commonly found in raw milk (Tzanetakis 1992), has been found to be effective against Listeria monocytogenes (Cleveland et al. 2001). The prevalence of Listeria monocytogenes in retail milk cheeses ranged from 0.9% at 2004 in the UK (Little et al. 2008) to 42.4% in Sweden during 1994 (Loncarevic et al. 1995), but Debuyser et al. (2001) found a higher incidence of Listeria monocytogenes in cheeses made from pasteurized (8%) versus raw (4.8%) milk. In Greece, the prevalence of Listeria monocytogenes in ovine and caprine herds that were suspected to suffer from meningocencephalitis was 19.3 and 36.9%, respectively (Giannati-Stefanou et al. 2006). In addition, the predominant serotype was 4b, which was isolated from curd in this study.

E. coli O157:H7 is a food pathogen that has been associated with severe food poisoning outbreaks involving meat products and raw milk cheese. The enteropathogenic E. coli O157: H7 was not detected in cheeses prepared with raw and pasteurized milk, and the products were considered to be safe. However, studies examining the survival of the pathogen in hard cheese indicate that the pathogen can grow during cheese manufacturing and can survive for up to 70 days after manufacturing (Hudson et al. 1997). Therefore, the indications are that the hardness imposed during manufacturing is insufficient to prevent growth of the pathogen in cheese products from milk contaminated with the pathogen. In addition, studies on the survival of the pathogen in feta and teleme cheeses (Govaris 2002) have found that E. coli O157: H7 survives the manufacture and ripening period. In recent years, several outbreaks of E. coli O157: H7 have been linked...
to the consumption of cheese in England (Strachan et al. 2001), in France (Espie et al. 2006) and in the Czech Republic (Bielaszewska 1997). Although pathogens were not detected in this work, the sporadic and seasonal occurrence of the pathogen in the feces of small ruminants (Kudva et al. 1996) without any symptoms, the low infection dose in humans and its survival in cheeses ripened for a long period of time suggest that this pathogen constitutes a threat to the public. Furthermore, in Greece, 9.58% of raw ewes’ and goats’ milk was found to be positive for *E. coli* O157:H7 (Zdragas et al. 2009).

The results of this study indicate that *Staphylococcus aureus* reaches non-detectable numbers after the 60th day of the ripening period, but the high numbers of these bacteria in raw milk cheese indicate the poor hygienic condition of milk. The same results were referred by Zarate et al. (1997) in Tenerife goats’ milk cheese and in raw goats’ milk lactic cheese in France (Vernozy-Rozand 1998).

Few outbreaks of Salmonellosis due to the consumption of raw milk cheese have been recorded (De Valk et al. 2000) concerning soft or semi-hard cheeses. It is generally believed that Salmonella is of minor importance for human health in hard cheeses like ‘graviera’, moreover, the pathogen was not detected in the present study in raw or pasteurized milk cheese.

*Enterococci* can survive the ripening period due to their tolerance to salt and acidic conditions and they can, also, survive in traditional fermented cheeses, such as kefalotyri and Feta (Litopoulou-Tzanetaki 1990, Litopoulou-Tzanetaki and Tzanetakis 1991). In recent years, strains of *E. faecium* and *E. faecalis* were found to be resistant to vancomycin and they have been recognized as major nosocomial pathogens that are responsible for a variety of infections. Studies on VRE epidemiology support the hypothesis that animals are carriers of VRE (Garica et al. 2005). The potential risk of VRE after the consumption of cheese is low, particularly in Graviera cheese, where no isolates were detected in either pasteurized or raw milk.

Taking into account the results of this survey, it is concluded that ‘Graviera’ cheese produced with raw milk is as safe as the product from pasteurized milk. Raw milk ‘Graviera’ cheese is more attractive to consumers because of the superior flavour and higher organoleptic characteristics, but further measures should be implemented to minimize the potential growth and propagation of pathogenic bacteria, such as the improvement of hygienic conditions of raw milk production, cheese making and aging.

**REFERENCES**


