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

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■ Παρουσία των παθογόνων βακτηρίων στη «Γραβιέρα Κρήτης» που παρασκευάζεται από νωπό και παστεριωμένο γάλα

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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 log₁₀MPN/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

ΠΕΡΙΛΗΨΗ. 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. Εξετάστηκαν 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 *Escherichia coli* πριν από το στάδιο της αναθέρμανσης της τυρομάζας, ήταν 3-3,5 log₁₀MPN/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 ωρίμανσης του τυριού και στους δύο τύπους τυριών. Η *Listeria monocytogenes*, τύπος 4b απομονώθηκε στα τυριά δύο πειραματικών τυροκομήσεων και επιβίωσε σε αυτά μέχρι το στάδιο παραγωγής μετά την αλάτιση. Στα τυριά αυτά μετά το στάδιο της αλάτισης όπως και σε όλα τα υπόλοιπα παρέμεινε σε μη ανιχνεύσιμους αριθμούς. Στο τέλος της περιόδου ωρίμανσης όλα τα βακτήρια που εξετάστηκαν είχαν μειωθεί σε ασφαλή για τον καταναλωτή επίπεδα.

Λέξεις ευρετηρίασης: Γραβιέρα Κρήτης, νωπό γάλα, παθογόνα βακτήρια

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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 Agrafo, 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 as 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*) is small and varies according to the type of cheese that is being produced. Experimental inoculation of pathogens to raw milk at levels ranging between 10^4 and 10^5 cfu/ml during the production of hard and semi-hard cheese could result in no detection of pathogens after one day of the production process in Swiss hard cheese, except *Listeria monocytogenes* and low numbers of *Staph. aureus* (Bachmann and Spahr 1995). *Listeria monocytogenes* could be a potential risk for Graviera cheese prepared from raw milk, because survival of the pathogen in low numbers is referred to in the core at the end of the aging period (Samelis 2009). Many factors are implicated in the microbiological quality of raw milk hard cheeses, but cooking temperature and low pH after pressing resulted in the elimination of pathogens (Bachmann and Spahr 1995).

The main advantage of milk pasteurization is the protection of public health from foodborne pathogens, but pasteurization, also, assures the consistency and quality of the products (Donnelly 2001). On the other hand, manufacturing and consuming cheeses made with raw milk is faster and produces more pronounced flavour, due to the indigenous microflora and the diversity in this flora which contributes to the great differences in the organoleptic characteristics found in raw milk cheese (Abriouel et al. 2008).

Although the debate concerning the use of raw or pasteurized milk is ongoing, the reduction of pathogenic bacteria in the two types of Graviera cheese was studied in field trials in a small plant in Crete, where crucial factors such as local flora from raw milk and plant environment were implicated. The aim of this study was to evaluate the microbiological safety of "Graviera Kritis" made with raw milk under local and traditional conditions in comparison to the same cheese made with pasteurized milk.

MATERIALS AND METHODS

Manufacturing of cheese samples

The production of 'Graviera' cheese was carried out under local conditions in Crete with both raw and pasteurized milk. Three different cheese productions were carried out for each type of cheese. The traditional technology was used in the production of 'Graviera Kritis' and it was exactly the same for the two types of cheeses except for the starter culture that was added in pasteurized milk.

Pasteurized (72°C, 15 sec) and cooled (4°C) ewe's milk was warmed at 35°C in a stainless steel vessel and a starter culture (mesophilic and thermophilic or fresh yoghurt, 0.5%, pH 4.3) was added. After 30 min, the quantity of rennet (Ha-La Hansen's Lab., Copenhagen, DK) 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 was initially cooked by slow cooking (1°C/ 2½ min) until a temperature of 43-44°C was reached and then by fast cooking (1°C/1-1½ min) until a temperature of 50°C was reached. Stirring was 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 (NaCl percentage x 100)/ 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 ± 0.15 and $1.64 \pm 0.18 \log_{10} \text{MPN/g}$ 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 $1.10 \pm 0.63 \log_{10} \text{MPN/g}$, respectively). The *Escherichia coli* populations (Table 1) at time 0 were 3.72 ± 0.75 and $0.70 \pm 0.23 \log_{10} \text{MPN/g}$ 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 ± 0.39 and $0.46 \pm 0.00 \log_{10} \text{MPN/g}$ 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 $1.19 \pm 0.39 \log_{10} \text{cfu/g}$ 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 *CoPS*¹ populations during the processing of Graviera cheese prepared with raw (R) and pasteurized (P) milk (log MPN/g)

Production stage	Day	<i>Coliforms</i> (log MPN/g)		<i>E. coli</i> (log MPN/g)		<i>S. aureus</i> (log cfu/g)	
		R	P	R	P	R	P
Before cooking	0	5.07 ^c ±0.15	1.64 ^a ±0.18	3.72 ^{gi} ±0.75	0.70 ^e ±0.23	4.08 ^m ±0.20	1.19 ^l ±0.39
After cooking	2h	4.62 ^c ±0.51	1.90 ^a ±0.29	3.21 ^g ±0.58	1.20 ^e ±0.43	3.16 ^m ±0.64	1.08 ^l ±0.31
Before salted	1	5.38 ^{bc} ±1.00	4.71 ^{bc} ±0.66	5.38 ^{fi} ±1.00	4.65 ^{fi} ±1.07	4.33 ^{ml} ±1.22	2.54 ^{lm} ±1.24
After salted	3	5.50 ^{bc} ±0.72	5.24 ^{bc} ±0.50	4.72 ^{fgi} ±0.88	5.21 ^{fgi} ±0.67	4.23 ^{mk} ±1.22	4.35 ^{km} ±0.64
Ripening	60	3.87 ^{bc} ±0.50	5.24 ^{bc} ±0.72	3.87 ^{gi} ±0.50	4.32 ^{gi} ±1.22	0.46 ⁱⁿ ±0.00	0.46 ⁱⁿ ±0.00
	90	2.90 ^{bc} ±0.23	3.93 ^{bc} ±0.33	2.90 ^{ig} ±0.23	3.93 ^{ig} ±0.72	0.46 ⁱⁿ ±0.00	0.46 ⁱⁿ ±0.00
Storage	120	1.10 ^{ad} ±0.63	1.01 ^{ad} ±0.41	0.46 ^{eh} ±0.00	0.85 ^{eh} ±0.39	0.46 ⁱⁿ ±0.00	0.46 ⁱⁿ ±0.00

^{a-m} Means within the same characteristics with different superscripts that differ significantly (P<0.05)

¹ 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.

Cheese type		Days					
Time		Before cooking	After cooking	Before salting (1 day)	After salting (4 days)	45	90
pH	R	6.27 ^c ±0.23	6.48 ^c ±0.15	5.3 ^{ab} ±0.049	5.38 ^b ±0.075	5.38 ^b ±0.2	5.07 ^a ±0.079
	P	6.76 ^d ±0.04	6.77 ^d ±0.02	5.97 ^c ±0.16	5.94 ^c ±0.11	5.52 ^b ±0.29	5.11 ^a ±0.23
Moisture %	R	67.1 ^d ±4.15	53.69 ^c ±9.48	45.51 ^{bc} ±3.008	41.92 ^{ab} ±4.14	39.44 ^{ab} ±1.1	33.6 ^a ±1.96
	P	67.42 ^c ±5.83	53.56 ^c ±5.41	47.2 ^{cd} ±4.83	42.92 ^{bc} ±3.37	36.85 ^{ab} ±8.45	31.52 ^a ±3.46
NaCl-in-moisture %	R	ND	ND	ND	3.05 ^a ±0.82	2.53 ^a ±0.28	2.417 ^a ±0.14
	P	ND	ND	ND	3.41 ^a ±0.688	3.37 ^a ±0.538	3.128 ^a ±0.209

^{a,b,c,d} 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

detected after the 60th day. (Table 1).

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 log₁₀ 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 abundance 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 Bergkasee 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* (Clevelant et al. 2001). The prevalence of *Listeria monocytogenes* in retail raw 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 meningoencephalitis 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 (DeValk 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 (Garcia 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. ■

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