PAPER

Field handling conditions of raw milk sold in vending machines: experimental evaluation of the behaviour of *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella Typhimurium* and *Campylobacter jejuni*

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Abstract

The direct sale by farmers of raw milk for human consumption has been allowed in Italy since 2004. The aim of this study was to evaluate the behaviour of selected foodborne pathogens in raw milk sold in vending machines, in field handling conditions, and during shelf-life from production to consumption. Temperature of storage of raw milk in 33 farms authorized to produce and sell raw milk were investigated from farm to vending machine delivery, together with consumer habits in one province of the Emilia-Romagna region of northern Italy. Failure to maintain appropriate low temperatures during shelf-life was recorded and 43% of consumers did not boil milk before consumption. *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella Typhimurium* and *Campylobacter jejuni* strains were inoculated into raw milk samples, and the best (4°C as established by law) and worst temperature storage conditions detected (variable temperature) were simulated. Boiling tests were performed for each pathogen considered at high and low levels of contamination. Results showed an increase in *L. monocytogenes* in milk stored at 4°C and at variable temperatures recorded in shelf-life monitoring, an increase in *E. coli* O157:H7 and *S. Typhimurium* at variable temperatures but not at 4°C, and a decrease in *C. jejuni* in all storage conditions. Boiling milk is effective in making it safe for consumers.

This study provides evidence that appropriate handling of raw milk, maintaining low temperatures, together with consumer education concerning boiling raw milk before consumption are key factors in preventing foodborne infections linked to raw milk consumption, and helps assess the risk of foodborne infection linked to raw milk consumption.

Introduction

The direct sale by farmers of raw milk for human consumption has been allowed in Italy since 2004. Many people are consuming raw milk in line with a desire to purchase local products and consume natural unprocessed food, greater freedom of choice, and the promotion of raw milk by certain groups (Oliver *et al.*, 2009). Another apparent reason is that raw milk is less expensive to buy than pasteurized retail milk. To meet the demand for *produce, sell and buy local*, farmers have increased their sales with automatic self-service vending machines located on farms, in cheese factories, in front of supermarkets, in public squares, in car parks or along crowded high streets. The vending machines sell raw milk and, usually, polyethylene (PET) or glass bottles, so consumers can buy the bottles or use their own.

According to Italian law, raw milk sold in vending machines must be refrigerated at less than 4°C as soon as possible after milking and maintained at this temperature during transportation and storage in vending machines until delivery to the consumer. Milk in vending machines must be replaced every day, so that a batch of milk cannot stay in the vending machine more than 24 h. After a case report of hemolytic uraemic syndrome (HUS) related to the consumption of raw milk (Scavia *et al.*, 2009), the Italian Health Ministry published an ordinance (10 December 2008) establishing that vending machines should bear the notice *Milk must be boiled before consumption* and fixed the milk expiry date at three days after delivery to the consumer.

The presence of pathogenic bacteria in raw milk has been well documented both in Europe and in the USA, and the isolation rate varies considerably from study to study (Oliver *et al.*, 2005, 2009). *Listeria monocytogenes* and *Salmonella* spp. were the most commonly reported pathogens isolated from bulk tank milk from 2000 to 2009 (Oliver *et al.*, 2009).

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Key words: *Listeria monocytogenes, Escherichia coli* O157:H7, *Salmonella* spp., *Campylobacter jejuni*, Raw milk.

Received for publication: 18 August 2011.
Revision received:13 December 2011.
Accepted for publication: 11 January 2012.

Italian Journal of Animal Science 2012; 11:e24
doi:10.4081/ijas.2012.e24

Surveys on foodborne pathogens in raw milk in Italy were basically linked to official monitoring of the vending machines. Bertasi *et al.* (2008) reported prevalences of 0.08% for *Escherichia coli* O157:H7, 0% for *Salmonella*, 0.87% for *Listeria* spp. and 0.37% for thermotolerant *Campylobacter* in official controls carried out in 2007. The data reported by D'Amico *et al.* (2008) and Waak *et al.* (2002) on pathogens in raw milk suggest that the level of contamination (*L. monocytogenes*, *E. coli* O157:H7), if present, is extremely low, ranging from less than 1 to 60 CFU mL^{-1} for *L. monocytogenes* and less than 1 mL^{-1} for *E. coli* O157:H7. Van Kessel *et al.* (2004) estimated the contamination by *Salmonella* and *L. monocytogenes*, respectively, in 22 and 32 out of 861 bulk tank milk samples at levels of 1 to 40 CFU 10 mL^{-1}, while Humphrey and Beckett (1987) reported a *Campylobacter jejuni* contamination level of 16±30 CFU mL^{-1} in 9 out of 111 bulk tank milk samples analyzed.

Inappropriate storage temperatures and equipment sanitation could result in bacterial growth and a potential risk increase to consumers. Furthermore, improper handling of milk by consumers and their failure to boil milk before may further increase the risk.

The aim of this study was to evaluate: i) the temperature conditions of raw milk sold in vending machines during its shelf-life from production to consumption in one province of the Emilia-Romagna region in northern Italy; ii) the behaviour of selected foodborne pathogens in raw milk at the temperature conditions estab-

lished by law in the worst conditions of handling recorded and after boiling. These data serve to estimate the risk for consumers associated with consumption of raw milk.

Materials and methods

A preliminary survey on consumer habits, temperature conditions and transport duration of raw milk was carried out to design an experimental trial which could evaluate the behaviour of selected foodborne pathogens in raw milk sold in vending machines during shelf-life from production to consumption.

Consumer interview

In summer (June/July) 2010, 100 raw milk consumers were interviewed while purchasing raw milk at vending machines on their habits regarding the use of insulated bags to transport raw milk home, the mean duration of transportation, and whether they boiled the milk before consumption. The results of the interview were used to design the inoculation test.

Temperature conditions of raw milk

All 33 farms authorized to produce and sell raw milk in the province were considered. The province was taken as an epidemiological unit because the direct sale of raw milk is allowed only for the local area, i.e. the province of production of the raw milk and the neighbouring provinces. The 33 farms served 60 vending machines (1-7 vending machines per farm) and together sold about 3800 L of raw milk daily.

In 2010, in collaboration with public veterinary services, data on the temperature of bulk tank milk at the time of loading for transportation, throughout transportation from farm to vending machine, and the temperature of milk on delivery to the vending machines were recorded in all the farms and for each vending machine considered. Temperature data were collected by a Delta OHM HD 9214 thermometer (resolution 0.1°C, calibrated by SIT, National Calibration Centre) for bulk tank milk and vending machines, and by Hobo H08-002-02 data logger (resolution 0.1°C, calibrated by SIT, National Calibration Centre) every 3 min throughout transportation. Temperature monitoring was repeated twice: in winter (January/February) and in summer (June/July). Time from farm to the vending machine was also recorded. On the basis of the results of the consumer interview, a simulation was carried out in July 2010 by buying one litre of milk at the vending machine and transporting it for 30 min by car without air conditioning and without using an insulated bag. Air and milk temperature during transportation were monitored using a Hobo H08-002-02 data logger (resolution 0.1°C, calibrated by SIT, National Calibration Centre). The simulation was repeated on four different days.

The results of the simulation trial, together with data on temperature conditions of raw milk from farm to vending machine, time of transportation and the temperature of milk on delivery to the vending machines were used to design the experimental trial.

Testing the behaviour of pathogens in raw milk at different temperature conditions

The following tests were repeated for three batches of raw milk collected on the same day from the same vending machine.

Ten one litre bottles of raw milk were bought from a vending machine close to the laboratory, placed in a cool box (5°C±3) and taken to the laboratory within 10 min. On arrival, 25 mL of milk were sampled from each bottle, pooled and tested for the presence of *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* O157:H7 and thermotolerant *Campylobacter* using official cultural methods (ISO, 2001; 2002; 2004; 2006). In addition, inhibitory substances were determined by Delvotest SP (Tecnomilk®) and alkaline phosphatase was measured by fluorometric assay, using the Fluorophos® ALP Test System. The remaining milk in the bottles was used for the experimental contamination test.

Three different strains of each pathogen considered in this study were used to obtain the bacterial inocula for the artificial contamination of raw milk. Details on the strains are reported in Table 1. Suspensions of each strain of *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7 and *C. jejuni* were prepared and quantified spectrophotometrically. Suspensions of the three strains were mixed and used to inoculate raw milk to obtain a final concentration of approximately 50-100 CFU mL^{-1} to simulate a low concentration of the pathogen. Two bottles were inoculated with each pathogen and the remaining two bottles (not inoculated) were used as controls to evaluate and compare the trend in pH and lactic acid population. Two groups (A and B) of five bottles (one bottle for each pathogen and one control) were created: group A was maintained at 4°C±0.5 throughout the test (96 h) to simulate the best conditions of storage; group B was stored at different temperatures to simulate the worst conditions detected in the preliminary survey, i.e. 7.0°C±0.5 for 5 h (maximum temperature registered during the transport from farm to the vending machine and transport duration), then at 11°C±0.5 for 22.5 h (maximum temperature registered in vending machines and maximum storage time established by law for raw milk), 30°C±0.5 for 30 min (worst air temperature during the simulation of transport of raw milk from vending machines to the home in summer) and 12°C±0.5 for 68 h (data obtained by Beaufort *et al.* 2008 as simulation of home storage).

At the end of the trial (96 h), 250 mL (approx. a cupful) of milk from all bottles was boiled. During boiling, milk temperature was monitored using a Delta OHM HD 9214 thermometer (resolution 0.1°C, calibrated by SIT, National Calibration Centre).

A further boiling test was performed on a different batch of raw milk with high contamination inoculum. The four bacterial suspensions were prepared as described above and each was inoculated into one litre of raw milk at a high concentration level (10^7-10^8 CFU mL^{-1}). A further 25 mL of milk were collected from all samples after boiling (both at low and at high inocula level), to perform a presence/absence test with cultural ISO methods (ISO, 2001, 2002,

Table 1. Strains used for raw milk inoculation.

	Strain
Listeria monocytogenes	ATCC 6994 Wild strain isolated from raw milk DUP 1042 (IZSLER) Wild strain isolated from raw milk DUP 1045(IZSLER)
Campylobacter jejuni	ATCC n. 49943 Wild strain isolated from raw milk FED 2010/31(DSMV) Wild strain isolated from raw milk FED 2010/111(DSMV)
Escherichia coli O 157:H7	ATCC 35150 Wild strain isolated from raw milk DUP 3064(IZSLER) Wild strain isolated from raw milk DUP 18588(IZSLER)
Salmonella Typhimurium	ATCC 6994 Wild strain isolated from raw milk 2010/67492 (IZSLER) Wild strain isolated from raw milk 2010/67498 (IZSLER)

2004, 2006).

During the trial, samples of raw milk were collected from each bottle immediately after inoculation (T0) and at the end of each phase: after 5 h (T1), 27.5 h (T2), 28 h (T3), 96 h (T4) and after boiling (T5), as listed in Table 2. Each sample was analyzed by plating 0.2 mL in five plates (in triplicate) of selective medium for enumeration of specific pathogens: ALOA agar (Oxoid) incubated at 30°C±1 for 48 h was used for *L. monocytogenes*, Hektoen agar (Oxoid) incubated at 37°C±1 for 24 h for *S. Typhimurium*, O157:H7 CT-SMAC agar (Oxoid) incubated at 37°C±1 for 24 h for *E. coli* and mCCD agar (Oxoid) incubated in a microaerobic atmosphere at 42°C±1 for 48 h for *C. jejuni*. At the same times (T0, T1, T2, T3, T4 and T5), each sample underwent further analysis as follows: *mesophilic lactobacilli* were counted by decimal dilution and inclusion in MRS agar plates (Oxoid) incubated under microaerophilic conditions at 35°C±1 for 48 h; *lactococci* were counted by decimal dilution and inclusion in M17 (Oxoid) agar plates incubated at 35°C±1 for 48 h; pH value was measured by an instrument with automatic temperature compensation (Hanna Instruments HI 223). A total of 9 analyses for each determination were performed (3 batches for 3 replicates). Data were normally distributed and were calculated as mean±standard deviation.Data were processed and fitted using ComBase Predictor programs (Dmfit) available on www.combase.cc and based on Baranyi model (Baranyi and Roberts, 1994; Baranyi and Tamplin, 2004).

Results

Consumer interview

Out of 100 consumers interviewed, 82% did not use insulated bags to transport raw milk to the home, while 4% only used them in summer; 14% of consumers always used insulated bags to transport raw milk to the home. Duration of transportation home ranged from a few minutes to 30 min (average 18 min). Forty-three percent of consumers did not boil milk before drinking (23% drank raw milk and 20% heated the milk in the microwave without reaching boiling point) and 57% of consumers boiled the raw milk before consumption.

Time and temperature controls

Duration of transportation from farm to vending machines varied widely from ten minutes to five hours. The worst temperatures of the bulk tank milk at the time of loading for transportation was 7.0°C. A maximum increase in temperature during transportation of 2.0°C (from 5.0°C at the moment of loading to 7.0°C on arrival at the vending machine) was recorded. The temperature of 7.0 °C was chosen to simulate the worst transportation condition from farm to vending machine. The higher temperature recorded by monitoring of milk delivered to the vending machines was 11.4°C. During the simulated transportation to the home, air temperature ranged from 28.5 to 30.1°C; the maximum increase in milk temperature was 5.5°C (from 6.2°C to 11.7°C) after 30 min.

Behaviour of pathogens in raw milk at different temperature conditions

No pathogens were found in any sample of raw milk tested before inoculation. All samples met the requirements for alkaline phosphatase (constantly above 350,000 mU/mL) indicating that milk used for the inoculation test had not been heat-treated and no inhibitory substances were detected. Table 2 lists quantities of the four pathogens during the storage tests under the best and worst storage conditions. There was an increase in *L. monocytogenes* in both storage conditions. From an initial value of 2.23±0.03 log CFU mL^{-1}, *L. monocytogenes* reached a final count of 2.61±0.02 log CFU mL^{-1} when stored at 4°C and from an initial value of 2.18±0.03 log CFU mL^{-1} reached a final count of 3.25±0.31 Log CFU mL^{-1} when stored at variable temperatures. The calculated doubling time for *L. monocytogenes* was 69 h 41 min and 27 h 39 min at 4°C and at variable temperatures, respectively. From an initial value of 2.14±0.12, *E. coli* O157:H7 grew to a final count of 3.97±0.28 log CFU mL^{-1} when stored at variable temperatures. The doubling time for *E. coli* O157:H7 was 10 h 57 min at variable temperatures. When stored at 4°C, *E. coli* O157:H7 count remained substantially unchanged from an initial value of 2.40±0.09 to a final value of 2.10±0.13. *C. jejuni* log CFU mL^{-1} count decreased from an initial value of 1.92±0.06 to a final value of 1.72±0.07 when stored at 4°C and of 1.28±0.19 when stored at variable temperatures; the calculated decimal reduction time (DRT) was 624 h 19 min at 4°C and 132 h 39 min at variable temperature conditions. *S. Typhimurium* count did not change when stored at 4°C, showing a slight decrease from 1.98±0.19 log CFU mL^{-1} to 1.85±0.25 log CFU mL^{-1}, but showed an increase from 1.88±0.09 log CFU mL^{-1} to 3.20±0.06 log CFU mL^{-1} when stored at variable temperatures.

The temperature reached during each boiling test (mean of 3 repetitions ±SD) ranged from 69.33±1.15°C to 71.66±2.88°C; no viable pathogenic bacteria were recovered from boiled milk either by direct plating or by enrichment procedures from samples inoculated at low and high inoculation levels.

Profile of pH and lactic acid bacteria

The initial (T0) pH value was equal to 6.69±0.04 SD and progressively decreased to values at T4 of 6.50±0.02 SD in milk stored at

Table 2. Pathogenic microbial population count during storage at 4°C and at variable temperatures (mean±SD Log CFU mL1 of 9 data: 3 replicates ×3 batches).

		Listeria monocytogenes		*Escherichia. coli* O 157:H7		*Campylobacter jejuni*		*Salmonella Typhimurium*	
Phase	Sampling, total time	4°C±0.5	Variable conditions	4°C±0.5	Variable conditions	4°C±0.5	Variable conditions	4°C±0.5	Variable conditions
Inoculation	T0, 0 h	2.23±0.03	2.18±0.03	2.40±0.09	2.14±0.02	1.92±0.06	1.92±0.06	1.98±0.19	1.88±0.09
Transport to the vending machine°	T1, 5 h	2.29±0.41	2.30±0.11	2.42±0.05	2.28±0.14	1.82±0.25	1.98±0.01	1.77±0.12	1.94±0.10
Storage in the vending machine#	T2, 27.5 h	2.35±0.20	2.45±0.24	2.17±0.16	3.15±0.30	1.74±0.13	1.97±0.07	1.78±0.11	2.00±0.01
Transport home§	T3, 28 h	2.41±0.06	2.50±0.13	2.28±0.09	2.90±0.02	1.84±0.06	1.96±0.07	1.51±0.07	2.72±0.24
Home storage^	T4, 96 h	2.61±0.02	3.25±0.31	2.10±0.13	3.97±0.28	1.72±0.07	1.28±0.18	1.85±0.25	3.20±0.06
After boiling	T5, 96 h	nd	nd	nd	nd	nd	nd	nd	nd

°Time and temperature of incubation in each phase during storage at variable temperatures: °5 h at 7°C±0.5; #22.5 h at 11°C±0.5; §30 min at 30°C±0.5 air temperature; ^68 h at 12°C±0.5; nd, not detected.

4°C and to 5.16±0.05 SD in milk stored at variable temperatures. No significant differences were seen in inoculated and non-inoculated bottles (*data not shown*).

From a starting value of 6.38±0.13 log CFU mL^{-1}, an increase in Mesophilic *lactococci* was seen during the storage tests to 7.38±0.17 log CFU mL^{-1} in milk stored at 4°C and to 8.77±0.05 log CFU mL^{-1} in milk stored at variable temperatures. The population of *Mesophilic lactobacilli* showed a starting value of 5.34±0.2 log CFU mL^{-1} and an increase to 5.71±0.09 log CFU mL^{-1} in milk stored at 4°C and to 8.70±0.10 log CFU mL^{-1} in milk stored at variable temperatures. No significant differences were seen in inoculated and uninoculated bottles (*data not shown*).

Discussion

The main evidence of the risk associated with raw milk consumption emerges from the increase in *L. monocytogenes* counts both at refrigeration temperature and under variable temperature conditions of storage, and from the multiplication of *S. Typhimurium* and *E. coli* O157:H7 at variable temperature conditions of storage.

The slight decrease in the *E. coli* O157:H7 population in samples stored at 4°C for four days is in agreement with previous studies (Arias *et al.*, 2001; Wang *et al.*, 1997; Heuvelink *et al.*, 1998). The growth of 1.83 log CFU mL^{-1} during 96 h of storage at variable temperatures reported in our study is difficult to compare with previous studies performed at constant temperature conditions and for longer times. Wang *et al.* (1997) reported a 1-2 log CFU mL^{-1} growth in raw milk during four days of storage at 8°C and Massa *et al.* (1999) a 1-2 log increase after 72 h incubation in raw milk at 8°C in two out of seven strains tested. The total final effect of temperature (at variable temperature conditions) on growth rate of *E. coli* O157:H7 strains used in our study was similar to the effect of storage at a constant temperature of approximately 8°C. The decrease in pH observed during storage (approx. 1.5 units) and the increase in competitor microflora are comparable with those observed by Wang *et al.* (1997). The *E. coli* O157:H7 strains used in our study also confirm the acid tolerance and good competitor ability (Massa *et al.*, 1999).

The ability of *L. monocytogenes* to grow at refrigeration temperatures is well-known, with reported minimum growth temperatures in milk of between -0.1 and -0.4°C (Walker *et al.*, 1990). Walker *et al.* (1990) found an increase of approximately 2 log CFU mL^{-1} in four days in UHT milk stored at 8.7°C. The same study reported a lag time of one day at 5°C and less than one day at 7.5 and 9.3°C, and a generation time of 20 h, 16 h and 5.5 h, respectively, at 5°C, 7.5°C and 9.3°C. Rosenow and Marth (1987) calculated a lag time of one day and a generation time of 11 h and 37 min in autoclaved milk stored at 8°C. The increase in *L. monocytogenes* count at variable temperature conditions in our study was 1.07 log CFU mL^{-1} in 96 h, but we did not design a sampling plan to assess the lag time and the generation time. The total effect of the variable temperature conditions of storage on the replication rate of *L. monocytogenes* led to a slower count increase with respect to values calculated on the basis of data reported by Walker *et al.* (1990) and by Rosenow and Marth (1987). The observed differences are probably due to the inhibitory effects of natural flora, namely lactic acid bacteria: Bovill *et al.* (2000) demonstrated a longer lag time and generation time of *L. monocytogenes* in pasteurized milk than in UHT milk and this effect could be greater in raw milk in which the competitor microflora is more abundant.

S. Typhimurium count was substantially unaffected by storage for 96 h at 4°C. *Salmonella*e have been reported to grow at temperatures below 5°C (D'Aoust, 1991), but the growth of most *Salmonellae* is prevented at temperatures below 7°C (Fares, 2007). The observed increase (1.32 log CFU mL^{-1}) in *S. Typhimurium* during storage at variable temperature conditions is comparable (1.20-1.69 log CFU mL^{-1}) to that reported in inoculated UHT milk after storage for 96 h at a constant 9°C temperature (Fares, 2007). The comparison of the growth rate obtained for E.coli O157:H7, *L. monocytogenes* and *S. Typhimurium* stored at variable temperatures with the data previously reported showed that a constant incubation temperature of 8-9°C seems to be suitable to simulate the field condition of raw milk handling to evaluate the growth rate of bacteria.

The decrease in *C. jejuni* count in both storage conditions is in agreement with previous reports (Doyle and Roman, 1982; Humphrey and Beckett, 1987). Doyle and Roman (1982) reported a high variability in decreasing trend among strains of *C. jejuni* in milk stored at 4°C. In addition, the inactivation of *C. jejuni* corresponded to an increase in competitor microflora, a decrease in pH and, probably, the activation of the lactoperoxidase system by H2O2 produced by bacteria growing in raw milk. This seems to be in agreement with our data in which the decrease in viable *C. jejuni* was greater in milk stored at variable temperatures (DRT 132 h 39 min.) than in milk stored at 4°C (DRT 624 h 39 min).

The presence of pathogenic bacteria in raw milk is sporadic but not uncommon. We previously isolated one *E. coli* O157:H7, one *C. jejuni* and one *S. Typhimurium* from three out of 99 raw milk samples collected from vending machines in the study province (Giacometti *et al.*, 2012). The present study has shown temperature abuse on different occasions during the shelf-life of raw milk that can lead to a replication of *L. monocytogenes*, *E. coli* O157 H7 and *S. Typhimurium* up to levels comparable with infectious doses. These doses are estimated to be 1-100 CFU for Shiga toxin-producing *E. coli* (Paton and Paton, 1998), as low as 100 cells for *Salmonella* depending on the food involved (Kothary and Babu, 2001), a dose of 500 organisms of *C. jejuni* is enough to produce symptoms (Black *et al.* 1988), while the infectious dose for *L. monocytogenes* still remains to be clarified given that it may vary according to the strain and consumer susceptibility. Our study estimated an increase of 1.07, 1.32 and 1.83 log CFU mL^{-1} for *L. monocytogenes*, *S. Typhimurium* and *E. coli* O157:H7 at variable temperature conditions. Hence, it may be assumed that at these temperatures accidental contamination can easily lead to pathogen multiplication up to infectious dose levels. Instead, for *C. jejuni* a decrease in the contamination level during shelf-life was observed both in samples stored at 4°C and at variable temperatures, although *C. jejuni* can survive at a low initial inoculation level. Considering that *campylobacter*s have been found in healthy cows at counts of 10^5 g^{-1} - 10^9 g^{-1} of faeces, only a few grams of faeces are needed to contaminate a bulk tank to produce a potentially infectious dose in a cup of milk (Yaman and Emali, 2004; Teunis *et al.*, 2005).

Conclusions

A constant temperature of approximately 8-9°C seems to be suitable to simulate the temperature field conditions of raw milk handling: this could be used for further tests on other foodborne pathogens. Inappropriate storage temperature of raw milk determines *E. coli* O157:H7, *Salmonella Typhimurium* and *Listeria monocytogenes* growth and a potential increase in risk for consumers. Farmers are expected to ensure optimal storage of raw milk. Boiling milk proved to be an effective tool for consumers to make the it safe, both at low and high contamination levels.

Unfortunately, 43% of the consumers inter-

viewed did not boil raw milk before consumption and raw milk is frequently drunk by children. Education about boiling raw milk before consumption is a key factor in reducing the risk of foodborne disease due to raw milk consumption.

The study contributes to the assessment of the risk of foodborne infection linked to consumption of raw milk sold from vending machines in Italy.

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