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SHORT COMMUNICATION:

***Arcobacter butzleri* and *Arcobacter cryaerophilus* survival and growth in artisanal and industrial ricotta cheese**

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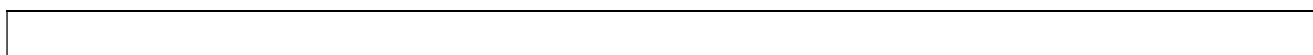
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Keywords: *Arcobacter butzleri*, *Arcobacter cryaerophilus*, ricotta cheese, survival, growth

Abstract

Ricotta cheese is a ready-to-eat product with properties (pH > 6.0, aw > 0.98-0.99) and moisture content (75-80%) that may pose a risk to public health due to post-process contamination by several bacterial pathogens, including *Arcobacters*. The objective of the study was to evaluate the behavior of *A. butzleri* and *A. cryaerophilus* in ricotta cheese during its shelf life assuming post-processing contamination. Two types of ricotta cheese, artisanal water buffalo (WB) and industrial cow's milk ricotta cheese, were experimentally contaminated with *A. butzleri* and *A. cryaerophilus* and the count was monitored at two different temperatures (6 and 12°C) during shelf life of 5 days for WB cheese and 22 days for industrial ricotta cheese. In WB ricotta cheese the *A. butzleri* count remained stable during the 5 days of storage at 6°C, whereas a moderate but significant (p=0.0068) decrease was observed in *A. cryaerophilus* count. The counts of both species increased when WB ricotta cheese was stored at 12°C. In industrial ricotta cheese stored at 6°C a significant reduction was observed both in *A. butzleri* and *A. cryaerophilus* counts during the 22-day storage period; at 12°C storage, a



count increase was observed for both *Arcobacter* species up to the 14th day of storage after which the log CFU/g count resulted constant until the 22nd day of storage. The ability of *A. butzleri* and *A. cryaerophilus* to survive at 6°C and to grow at 12°C in ricotta cheese have significant food safety implications.

MANUSCRIPT

Arcobacter species have a widespread distribution with a broad range of animal hosts, food and environmental reservoirs, and are increasingly associated with human illness (Doudah et al., 2014). Some *Arcobacter* species are considered emerging enteropathogens and potential zoonotic agents (Collado and Figueras, 2011). In particular, the species *A. butzleri* and *A. cryaerophilus* have been reported to infect humans. A recent study found that members of the *Arcobacter* genus were the fourth most common pathogenic group (after *Campylobacter* spp., *Salmonella* spp. and toxigenic *Clostridium difficile*) isolated from fecal samples from persons with acute enteric disease (Van de Abeele et al., 2014).

In the dairy chain, *Arcobacter* spp. have been isolated from fecal samples of dairy animals (Wesley et al., 2000; Golla et al., 2002; Van Driessche et al., 2005; Vilar et al., 2010; Piva et al., 2013; Shah et al., 2013), in-line milk filters (Serraino et al., 2013b) and cow and water buffalo milk (Scullion et al., 2006; Milesi, 2010; Shah et al., 2012; Yesilmen et al., 2014). Raw or minimally processed foods are usually considered the main source of human *Arcobacter* infection in industrialized countries, and in food of animal origin, the initial source seems to be fecal contamination during the various stages of production (Ongör et al., 2004; Scullion et al., 2006, Van Driessche and Houf, 2008). However, *Arcobacter* contamination of food processing surfaces has been reported in poultry slaughterhouses, spinach processing plants, and dairy plants (Houf et al., 2002; Houf et al., 2003; Gude et al., 2005; Son et al., 2006; Ferreira et al., 2013; Giacometti et al., 2013a and b; Hausdorf et al., 2013; Serraino and Giacometti, 2013; Scarano et al., 2014). Food processing surfaces were demonstrated to be a source of secondary contamination even for strongly processed foods and *A. butzleri* was isolated in

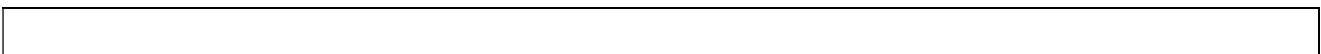
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both artisanal and industrial ricotta cheese at retail (Giacometti et al., 2013a; Scarano et al., 2014). These aspects pose a risk for consumers as ricotta cheese is a ready-to-eat product that provides a substrate ($\text{pH} > 6.0$, a_w 0.98-0.99 and moisture content at 75-80%) that is not limiting for the survival and growth of many pathogenic bacteria.

The objective of this study was to evaluate the behavior of *A. butzleri* and *A. cryaerophilus* in ricotta cheese during its shelf life assuming post-processing contamination, to establish whether ricotta cheese can support the growth of these microorganisms. Two types of ricotta cheese, artisanal water buffalo (WB) and industrial cow's milk ricotta cheese, were used for the experimental inoculation and the behavior of the *A. butzleri* and *A. cryaerophilus* populations was monitored at two different temperature conditions (6 and 12°C).

The artisanal WB ricotta cheese was produced in the cheese factory of the Department of Veterinary Medical Sciences, Bologna, Italy: about 10 kg of ricotta cheese were collected by sterilized tools and immediately transported to the laboratory; the industrial ricotta cheese was received from a local industry; ten 1.5 kg packs of industrial cow ricotta cheese were transported to the laboratory. Both types of ricotta cheese were divided in 18 batches of 500 g each and used for the experimental inoculation on the same day of production for WB ricotta cheese and on the day after production for industrial cow ricotta cheese. The shelf life declared by manufacturers was 5 and 22 days respectively for WB artisanal and industrial cow's milk ricotta cheese at storage conditions of 6° C.

For the inocula, one reference strain (*A. butzleri* strain DSM 8739^T and *A. cryaerophilus* strain DSM 7289^T, Leibniz Institute DSMZ, Braunschweig, Germany) and two field isolates, respectively two *A. butzleri* (AB-61 and AB-83), isolated from dairy processing surfaces and from cheese (ricotta draining table and artisanal water buffalo ricotta cheese) in an artisanal dairy plant, and two *A. cryaerophilus* (AC-1 and AC-G29), isolated from WB feces and from processing surfaces (curd-cutting facilities) in an industrial dairy plant, were used in the study. Each strain was grown separately on nutrient agar supplemented with 5% laked horse blood (Oxoid, Basingstoke, UK) incubated at



30°C for 48 h microaerobically by evacuating 80% of the normal atmosphere and introducing a gas mixture of 8% CO₂, 8% H₂ and 84% N₂ into the jar. Colonies present in the plates were collected by a sterile swab and suspended separately in saline (NaCl 0.85%, VWR International, Milan, Italy). The suspensions obtained were cultured separately in 1 liter of *Arcobacter* broth (Oxoid, Basingstoke, UK) incubated in continuous agitation at 30°C for 48 h aerobically, then centrifuged at 3,000 g for 1 h; the pellets were resuspended separately in 1 liter of saline (NaCl 0.85%, VWR International, Milan, Italy). For the bacterial inocula, the three suspensions of both *A. butzleri* and *A. cryaerophilus* strains were quantified spectrophotometrically (600nm), and diluted in Butterfield's phosphate buffer to obtain approximately the same concentration of each strain ($OD_{600} = 0,665 \pm 0.02$, 0.044 ± 0.03 and 0.184 ± 0.02 , respectively for *A. butzleri* inoculated in cow industrial ricotta cheese at 6°C, *A. butzleri* in other tests and *A. cryaerophilus* inocula). For each bacterial species, an equal volume of the three suspensions was pooled and the mix was used to inoculate ricotta cheese (5 ml of suspension were inoculated in 500 g of ricotta cheese and manually mixed with a sterile spoon) to obtain a final concentration in ricotta cheese of about 10^4 - 10^6 CFU/g. The number of viable cells of the mix suspensions was verified by tenfold dilution and direct plating on nutrient agar supplemented with 5% laked horse blood (Oxoid, Basingstoke, UK) incubated at 30°C for 48 h microaerobically.

For each ricotta cheese type, the eighteen 500 g batches were used as follows: 6 batches were inoculated with *A. butzleri* (3 batches were stored at 6°C and 3 batches at 12°C); 6 batches were inoculated with *A. cryaerophilus* (3 batches were stored at 6°C and 3 batches at 12°C); 6 batches were not inoculated and used as control (3 batches were stored at 6°C and 3 batches at 12°C). Storage conditions at 6°C and 12°C were selected to simulate conditions of optimal storage and thermal abuse at home throughout the shelf life claimed by each producer; the ricotta cheese batches were stored into two refrigerated incubators ClimasLab CIR 400 and the temperature was measured by a Thermo Button 22L data logger (Astori Tecnica s.n.c.). In addition, for each trial (two types of ricotta cheese x three batches x two incubation temperatures) a control was performed by inoculating the same

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concentration of the two *Arcobacter* species into 200 mL brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK).

From each type of ricotta cheese, 3 samples were collected before the *Arcobacter* experimental inoculation to verify the absence of natural *Arcobacter* spp. contamination by enrichment procedure, as described by Houf et al. (2001). Briefly, 25 g of sample were inoculated into 225 ml of *Arcobacter* broth (Oxoid, Basingstoke, UK) supplemented with 5% laked horse blood (Oxoid, Basingstoke, UK) and a mix of cefoperazone (16 mg/L), amphotericin B (10 mg/L), 5-fluorulacil (100 mg/L), novobiocin (32 mg/L), and trimethoprim (64 mg/L) as a selective supplement. All antimicrobial substances were obtained as laboratory standard powders from Sigma (St. Louis, MO, USA). After 48 h of incubation, an aliquot of 10 µL of the enrichment broth was streaked onto selective *Arcobacter* agar plates prepared by suspending 24 g/L of *Arcobacter* broth (Oxoid, Basingstoke, UK) and 12 g/L of Agar Technical No. 3 (Oxoid, Basingstoke, UK) and supplemented with selective supplement as described above. The plates were incubated at $30 \pm 1^\circ\text{C}$ under microaerobic conditions and after 48 h of incubation were checked daily up to 5 days.

From each inoculated batch, at each day of storage for WB artisanal ricotta cheese (from 0 to 5) and at days 0, 1, 2, 3, 7, 10, 14, 17 and 22 for industrial cow's milk ricotta cheese, one samples of 10 g was collected to count, in duplicate, respectively *A. butzleri* and *A. cryaerophilus*, by direct plating of serial decimal dilutions onto selective *Arcobacter* agar plates prepared as described above and incubated at $30 \pm 1^\circ\text{C}$ under microaerobic conditions for 72 h. Colonies were counted and a selection of 10 colonies for each plate were subcultured and examined for presumptive identification such as growth under aerobic condition and cellular morphology. The DNA of at least 5 colonies from each plate was extracted using the REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, USA) and subjected to species confirmation by multiplex PCR (Doudah et al., 2010).

From non-inoculated batches 3 samples were collected throughout storage (same sampling times) and analyzed by enrichment procedure, as described above to check the absence of *Arcobater* spp.

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The count from inoculated BHI broths was performed in parallel (same sampling times) and with the same procedure described for ricotta cheeses.

The following analyses were additionally performed in single on each sample for each batch: mesophilic lactic acid bacteria (LAB) count by tenfold dilution and inclusion in M17 and MRS agar plates (Oxoid, Basingstoke, UK) incubated aerobically and under microaerobic conditions respectively at 35°C for 48 hours; pH values were measured by an instrument with automatic temperature compensation (Hanna Instruments HI 223); a_w was determined by AquaLab model series 3.

The *A. butzleri* and *A. cryaerophilus* counts and the pH and a_w measurements at different time of storage were analyzed by repeated measures ANOVA; PRISM 5.0 software was used. Statistical significance was set at $p < 0.05$.

The results of the study show that no *Arcobacter* spp. were detected in samples of ricotta cheese analyzed before inoculation or in any of the samples performed in non-inoculated batches during the study. Values of pH, a_w , and LAB count showed no significant differences between non-inoculated batches and batches inoculated with *A. butzleri* and *A. cryaerophilus* in WB artisanal or in industrial ricotta cheeses. The a_w observed values resulted unchanged until the end of the shelf life in the range 0.994-0.998 for a_w ; pH values resulted substantially unchanged in WB ricotta cheese stored for 5 days at 6°C but showed a significant decrease in WB ricotta cheese stored at 12°C for 5 days (from 6.12 ± 0.02 to 5.21 ± 0.34) and in industrial cow ricotta cheese both stored at 6°C and at 12°C (from 6.46 ± 0.01 to 5.88 ± 0.05 and from 6.47 ± 0.00 to 5.59 ± 0.03 respectively) for 22 days. The LAB population resulted always <30 CFU/g in industrial cow's milk both in ricotta cheese stored at 6 and 12°C as well as in WB artisanal ricotta cheese at 6°C till the 5th day of storage in which they raise to 2.02 log CFU/g whereas at 12°C LAB increased to 9.32 log CFU/g (tables 1a, 1b, 2a and 2b) at the end of 5 days of storage.

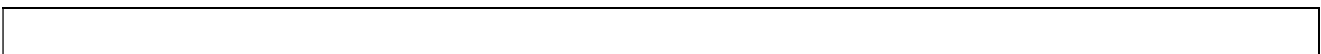
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In WB artisanal ricotta cheese the *A. butzleri* count remained stable during the 5 days of storage at 6°C (table 1a), whereas a moderate but significant ($p=0.0068$) decrease was observed in *A. cryaerophilus* count (between 0 vs 4 and 0 vs 5 days of storage). An increase was shown in the count of both species when WB ricotta cheese was stored at 12°C, from 4.27 ± 0.13 Log CFU/g to 8.03 ± 0.26 and from 5.15 ± 0.04 Log CFU/g to 8.34 ± 0.14 , for *A. butzleri* and *A. cryaerophilus* respectively (tables 1a and 1b). Values of pH showed a moderate increase during the storage period at 6°C and a significant decrease from the 4th day of storage in WB ricotta cheeses stored at 12°C to a value of 5.21 at the end of the 5-day period (see tables 1a and 1b). The LAB count, starting from a value of <30 CFU/g at day 0 resulted unchanged at 6°C until the 4th day of storage followed by an increase on the 5th day (tables 1a and 1b). In WB ricotta cheese stored at 12°C a significant increase in the LAB count was observed during the period at the 5th day of storage (tables 1a and 1b).

In industrial ricotta cheese stored at 6°C a significant reduction was observed both in *A. butzleri* and *A. cryaerophilus* count during the 22-day storage period (tables 2a and 2b); by contrast, at 12°C, a significant count increase was observed for both *Arcobacter* species up to the 14th day of storage (4.42 and 3.44 log CFU/g increase for *A. butzleri* and *A. cryaerophilus* respectively) after which the log CFU/g count slightly decreased until the 22nd day of storage (see tables 2a and 2b). A significant decrease in the pH value was observed in both ricotta cheeses stored at 6°C and at 12°C from the 7th day of storage as reported in tables 2a and 2b. The LAB count was < 30 CFU/g until the end of the storage time, see tables 2a and 2b.

In BHI broth, *A. butzleri* demonstrated the ability to replicate at 12°C and *A. cryaerophilus* count increased at both (6 and 12°C) the incubation temperatures chosen (data not shown).

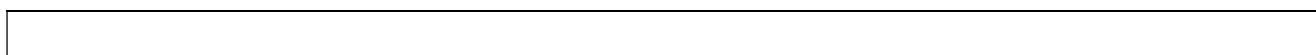
The present study is the first to investigate the behavior of *A. butzleri* and *A. cryaerophilus* in ricotta cheese. The results clearly show that ricotta cheese is able to support the growth of these *Arcobacter* species in case of moderate thermal abuse (12°C) and that both the investigated species are able to



survive during the shelf life of two different types of ricotta cheese when stored at refrigeration temperature (up to 22 days at 6°C).

The water activity and the pH resulted few or not limiting to *Arcobacter* growth or survival; in fact the a_w values was in the range 0.994-0.998 for all the period of the storage that is over the reported minimal a_w (0.985 to 0.990) for arcobacter growth (Cervenka, 2008). The pH values remained in the reported optimal pH range (6.0 to 8.0) (Lehner et al., 2005) for most of the study and in industrial ricotta cheese they never reached the lower pH growth (5.0 to 5.5) reported for *Arcobacter* (D'sa and Harrison 2005; Hilton et al., 2001) (see table 2). In artisanal WB ricotta cheese the pH reached a 5.21 value only at the 5th day of storage at 12°C.

A. butzleri count remained stable in artisanal WB ricotta cheese but decreased in industrial ricotta cheese when stored at 6°C (see tables 1a and 2a). A progressive decrease of the log CFU count was in agreement with several reports in cultural media (Hilton et al., 2001; Kjelgaard et al., 2009), water with or without organic material (Van Driessche and Houf, 2008) and milk (Giacometti et al., 2014) stored at refrigeration temperatures. Given that the same strains and the same storage conditions were used in the two experiments, the apparent variables between the two tests could be attributed to the different type of cheeses used in terms of composition (water buffalo vs cow's milk ricotta cheese) or in terms of natural contaminant microflora (artisanal vs industrial production system): both the composition of the medium (Kjeldgaard et al., 2009) and the presence of natural microflora (Balamurugan et al., 2013) were demonstrated to influence the growth and survival of *A. butzleri* in foods. In particular, Balamurugan et al. (2013) demonstrated that natural contaminant microflora may enhance *A. butzleri* survival on vacuum-packaged beef and they speculated that the enhanced survival could be attributed to the scavenging of oxygen passing through bag barrier (that can be excluded in the present study) or to the production of metabolites by the natural microflora. In addition, the reported decrease in cow industrial ricotta cheese, in comparison to WB artisanal ricotta cheese, could be due to the longer time (22 versus 5 days) the microorganisms are under unfavourable temperature

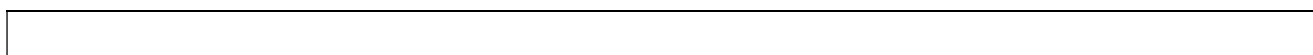


conditions and a similar trend could be observed in WB artisanal ricotta cheese if the test could be extended for a longer time.

A. cryaerophilus log CFU count decreased in the two types of ricotta cheese when stored at 6°C. The reported minimal growth temperature of *A. cryaerophilus* is 5°C (Neill et al., 1985) but the decrease observed in this study reflects a similar behavior in milk at 10 and 4°C (Giacometti et al., 2014).

When the ricotta cheeses were stored at 12°C a significant increase in both *A. butzleri* and *A. cryaerophilus* count was observed during the 5 days of storage in WB ricotta cheese (up to 8.03 and 8.34 log CFU/g respectively for *A. butzleri* and *A. cryaerophilus*) and in cow's milk ricotta cheese during the 22 days of storage (up to 8.69 and 8.06 log CFU/g respectively for *A. butzleri* and *A. cryaerophilus*). Kjelgaard et al. (2009) reported the minimum growth temperature for *A. butzleri* on chicken meat juice medium and BHI at 10°C. By contrast, both *A. butzleri* and *A. cryaerophilus* were unable to grow in milk at 10°C (Giacometti et al., 2014). The higher incubation temperature applied in the present study (12°C versus 10°C) and the different type of food tested may have influenced the results.

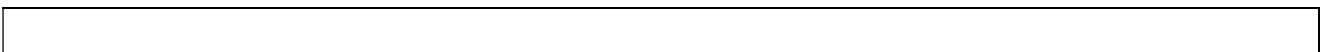
The ability of *A. butzleri* to survive during the shelf life of dairy produce in both cases of thermal abuse and optimal storage temperature was previously reported, but growth was observed only in the case of severe thermal abuse (Serraino et al., 2013a; Giacometti et al., 2014); the isolation of *A. butzleri* in ricotta cheeses sampled at retail and the ability of *A. butzleri* and *A. cryaerophilus* to grow at 12°C, i.e., roughly the temperature of home storage (Beaufort et al., 2008), can have food safety implications as ricotta cheese is a ready-to-eat product. In addition, the increase in *Arcobacter* spp. count in inoculated batches occurred without significant changes in organoleptic properties and pH in comparison to non-inoculated batches. This finding must also be taken into account as the consumer may have no indication of the multiplication of potential pathogenic bacteria in the cheese. The findings of this study should help to draw more attention to *A. butzleri* and *A. cryaerophilus* in dairy plants, and in the food processing environment in general, and to their importance as human



pathogens entering the food chains. Due to the few reports available in literature, future studies should address the retail prevalence of dairy products contaminated by *Arcobacter* spp. for the development of a proper risk assessment.

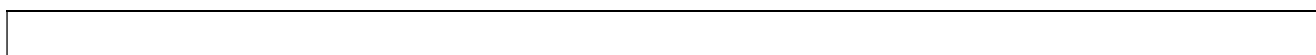
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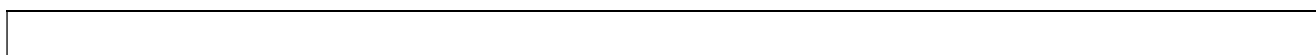
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