Strain- and serotype-dependent affinity of Shiga toxin-producing
Escherichia coli for bovine milk fat globules

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ABSTRACT

Shiga toxin-producing Escherichia coli (STEC) are widely detected in raw milk products intended for human consumption. Although STEC are a worldwide public health problem, the pathogenicity of STEC in cheese remains unclear. In fact, bacterial association with compounds in raw milk cheeses could reduce their pathogenicity. A previous study showed the association of 2 STEC strains with raw milk cream in a natural creaming assay. Different concentrations of each strain were required to saturate the cream. In this study, we hypothesized that all STEC strains could be associated with milk fat globules (MFG) in raw milk and that the bacterial load required for saturation of the cream is serotype dependent. We evaluated the affinity of STEC strains belonging to the O157:H7, O26:H11, and O103:H2 serotypes for bovine raw milk cream and analyzed saturation of the cream layer by natural creaming assay. We used 12 STEC strains and 3 strains belonging to another pathotype to assess the effects of serotypes on this phenomenon. We performed sucrose density gradient centrifugation assays with 2 STEC model strains to confirm the results obtained by natural creaming. The localization of STEC within MFG-enriched creams was observed by confocal and electron microscopy. We recovered approximately 10 times more STEC from the cream layer after natural creaming than from raw bovine milk. The concentration of STEC required to saturate the cream layer (the saturation concentration) was estimated for each strain by nonlinear regression, highlighting a strain and serotype effect. Moreover, the concentration of STEC in the cream was milk fat level dependent. However, even in nonsaturating conditions, a high level of STEC was still present in the aqueous phase, after fat separation. Thus, natural creaming should not be used as the sole preventive measure to remove STEC from naturally contaminated raw milk. The results of our study suggest that cream saturation is a complex mechanism, most likely involving specific interactions between STEC and raw MFG.

Key words: STEC, bovine raw milk cream, creaming, cream bacterial saturation, food microbiological safety

INTRODUCTION

Raw milk is a biological fluid produced by lactating mammals as a nutritional source for their young. It is an oil-in-water-type emulsion, consisting of an aqueous phase (>80%) in which lactose, whey proteins, and minerals are dissolved (Jost, 2007). The dispersed phase consists of casein micelles and milk fat globules (MFG), which account for at least 95% of the milk fat (Jost, 2007). Whole cow milk generally contains 35 to 40 g/kg of fat (Silanikove et al., 2015). Raw milk is slightly acidic or close to neutral pH and contains various ions. For example, bovine raw milk has a pH close to 6.7 to 6.8 and an ionic strength of 80 mM (Gauceron, 2005; Nian et al., 2012).

Fermented raw milk products, such as raw milk cheeses, are consumed worldwide (Baschali et al., 2017; Arias-Roth et al., 2022). In some countries, they are part of the gastronomic heritage and contribute to regional socioeconomic development (Soni and Dey, 2014; Bagel and Sergentet, 2022). Raw milks, and therefore raw milk cheeses, have a rich microbiota from various environmental sources that are partly responsible for their organoleptic properties (Quigley et al., 2013; Inter-
national Dairy Federation, 2016). However, pathogenic bacteria including *Campylobacter* spp., *Salmonella* spp., Shiga toxin-producing *Escherichia coli* (STEC), and *Listeria monocytogenes*, may contaminate raw milk if poor hygiene practices have been applied during milking (EFSA BIOHAZ Panel, 2015). Surveillance and implementation of good practices at different steps of cheese production help to reduce the risk of contamination (FAO/WHO, 2021).

Raw milk and raw milk cheeses have been associated with foodborne STEC infections in humans from different countries (Honish et al., 2005; Espié et al., 2006; Baylis, 2009; Mungai et al., 2015; Perrin et al., 2015; Currie et al., 2018; FAO/WHO, 2019; Treacy et al., 2019). When STEC-contaminated milk is used to produce raw milk cheeses, STEC may survive and be ingested by the consumer. Therefore, the presence of STEC strains in milk and raw milk cheeses is a very important and complex issue for microbiological risk assessment and may result in significant economic losses for the industry. Pathogenic STEC have the potential to cause severe forms of infection, such as hemorrhagic colitis, and systemic complications in the form of hemolytic uremic syndrome, due to the action of Shiga toxins. Severe STEC-associated disease is more common in patients under 5 yr of age. The major STEC virulence factor is the Shiga toxin, which is encoded by the *stx* gene (ANSES, 2017). In addition, the locus of enteroctye effacement is found in most human-pathogenic STEC strains.

However, dairy products, in particular MFG, can have positive effects on the immune system, in part due to their antimicrobial properties (Ofek et al., 2003; Claëys et al., 2013; Yoon et al., 2016;_donellou et al., 2018; Bagel and Sergentet, 2022). Bovine MFG are lipid droplets, with a mean diameter of 4 μm, formed by a core of triglycerides enveloped by a biological phospholipid triple membrane called the milk fat globule membrane (MFGM) (Kosmerl et al., 2021). The MFGM acts as an emulsifier, prevents coalescence of MFG, and protects triglycerides against lipase activity (Dewet-tinck et al., 2008). As a consequence of the mechanisms of milkfat secretion from mammary epithelial cells, the MFGM is a complex trilayered structure, comprising a monolayer of polar lipids derived from the endoplasmic reticulum and a bilayer of polar lipids originating from the apical plasma membrane of the mammary secretory cells (Keenan and Mather, 2006; Evers et al., 2008; Lopez, 2020). Thus, the outer bilayer of the MFGM contains diverse (glyco)-proteins and (glyco)-lipids on its surface (Reinhardt and Lippolis, 2006). These glycoconjugates form the glycocalyx and act as a source of specific bacterial and viral ligands (Spitsberg, 2005; Donellou et al., 2017b) that can vary during lactation (Wilson et al., 2008).

Bacterial affinity for MFGM surface components has been shown for various bacterial species, particularly for lactic acid and propionic bacteria (Brisson et al., 2010; Donellou et al., 2017b; Gomand et al., 2018; Guerin et al., 2018). The work reported in Donellou et al. (2018) examined the affinity of 2 STEC strains belonging to key serotypes (O157:H7 and O26:H11) for bovine raw cream. The affinity of these pathogenic strains for the bovine raw cream was limited. Cream layers were only saturated at high STEC concentrations in raw milk, and the 2 strains presented different saturation profiles. Based on these observations, we hypothesized that all STEC strains have an affinity for raw milk cream and MFG, and that this affinity is serotype dependent.

The objectives of this study were (1) to confirm the affinity of STEC for bovine raw milk cream using a large STEC strain collection; (2) to identify their localization in raw milk cream; and (3) to determine the possible effect of serotype and strain on this phenomenon.

During natural creaming, MFG spontaneously rise to the surface due to the difference in density between MFG and the aqueous phase of milk (Stokes’ law; Bird, 1991). We performed natural creaming assays of raw milk experimentally contaminated with 12 STEC strains and 3 strains belonging to another pathotype to evaluate the affinity of STEC for cream. We contaminated milk with each strain separately, at different levels, to assess any strain-specific effects of the saturation concentration (SC). The phenomenon of saturation of STEC was affined with 2 model STEC strains. In addition, we studied the physical congestion of the cream layer by STEC and the effect of milkfat content on the level of STEC in cream, and we characterized the STEC cells that did not associate with MFG.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Conditions**

A total of 12 STEC (E. coli with the *eae* and *stx* genes) and 3 attaching-effacing *Escherichia coli* (AECC) strains (E. coli with the *eae* gene) were selected from the collection of the French National Reference Laboratory (VetAgro Sup, Marcy-l’Etoile, France). Details of STEC and AECC collection used in this study are presented in Table 1. This study included O157 and non-O157 strains: O157:H7 (n = 5), O26:H11 (n = 5), and O103:H2 (n = 5). We paid particular attention to
Table 1. Shiga toxin-producing *Escherichia coli* and attaching-effacing *E. coli* (STEC/AEEC) strain collection

| Strain       | Intimin and shiga toxin genotype | Origin                  | Detailed origin1 | Isolation date | Reference                                      |
|--------------|----------------------------------|-------------------------|------------------|----------------|-----------------------------------------------|
| Serotype O157:H7 |                                  |                         |                  |                |                                               |
| SAKAI        | cae− stx1− stx2+                 | Human                   | HUS              | 1996           | Hayashi et al., 2001                          |
| EDL933       | cae+ stx1− stx2+                 | Human                   | HUS              | 1983           | Perna et al., 2001                           |
| 1044         | cae+ stx1+ stx2+                 | Dairy product           | Bovine raw milk cheese | 2012           | French national STEC reference laboratory2   |
| 5280-B       | cae+ stx1− stx2+                 | Dairy product           | Bovine raw milk cheese | 2019           | French national STEC reference laboratory     |
| 2044-A       | cae− stx1+ stx2−                 | Dairy product           | Bovine raw milk cheese | 2016           | French national STEC reference laboratory     |
| Serotype O26:H11 |                                  |                         |                  |                |                                               |
| 21765        | cae+ stx1− stx2+                 | Human                   | HUS              | 2005           | Galia et al., 2015                           |
| 11368        | cae− stx1+ stx2−                 | Human                   | HUS              | 2001           | Ogura et al., 2007                           |
| 103          | cae+ stx1− stx2+                 | Dairy product           | HUS              | 2012           | French national STEC reference laboratory     |
| 2157-A       | cae+ stx1+ stx2+                 | Dairy product           | Bovine raw milk cheese | 2018           | French national STEC reference laboratory     |
| 4315-A       | cae+ stx1+ stx2+                 | Dairy product           | Bovine raw milk cheese | 2019           | French national STEC reference laboratory     |
| Serotype O103:H2 |                                  |                         |                  |                |                                               |
| PMK5         | cae+ stx1+ stx2−                 | Human                   | HUS              | 1993           | Mariani-Kurdjian et al., 1993                  |
| 32306        | cae− stx1+ stx2−                 | Human                   | HUS              | 2011           | Mother and Child Hospital3                     |
| 1487-A       | cae+ stx1− stx2−                 | Dairy product           | Bovine raw milk cheese | 2018           | French national STEC reference laboratory     |
| 2503         | cae+ stx1− stx2−                 | Dairy product           | Bovine raw milk cheese | 2012           | French national STEC reference laboratory     |
| 445-14       | cae+ stx1− stx2−                 | Dairy product           | Bovine raw milk cheese | 2012           | French national STEC reference laboratory     |

1HUS = hemolytic uremic syndrome.
2French national STEC reference laboratory, VetAgro Sup, Marcy-l’Etoile, France.
3Mother and Child Hospital, Hospices Civils de Lyon, Bron, France.

the origin and virulence genes of the strains. For each sub-serotype, we used 2 strains isolated from human cases and 3 others from bovine raw milk cheese. We selected strains according to the presence of stx1 or stx2 (n = 4), or the absence of these genes (n = 1). The nonpathogenic *E. coli* K-12 str. MG1655 was also included in this study.

Bacteria were stored at −80°C in brain heart infusion (BHI, BioMérieux) supplemented with 30% glycerol. Bacteria were plated from glycerin-BHI frozen stock (−80°C) on Luria-Bertani agar plates (Oxoid) and incubated at 37°C. This procedure was performed for each experiment described herein. Shiga toxin-producing *E. coli* (cae+, stx+) are potentially highly pathogenic, and safety precautions must be taken when using these strains. If not otherwise specified, all experiments were performed in a biosafety level 3 laboratory.

### Bovine Raw Milk

Bovine raw milk was purchased either from the local market or directly from farms (Lyon and surrounding towns, France). Raw milk was stored at 4°C and used within 3 d. The fat content of raw milk and the cream layer were quantified by Gerber’s acid butyrometry method, according to the standard NF ISO 19662 (Agrolabs, Aurillac, France). For confocal laser scanning microscopy (CLSM) observations, milk from the morning milking was purchased directly from a farm.

### E. coli Saturation Test in Bovine Raw Milk Cream by Natural Creaming Assay

**Experimental Plan.** A diagram describing the experimental plan design is available in supplemental data (Supplemental Figure S1; http://dx.doi.org/10.17605/OSF.IO/K86TA; Bagel et al., 2022). For each *E. coli* strain, an adequate volume of overnight bacterial suspensions, containing approximately 5 × 10^9 cfu according to the optical density (OD600)/cfu per mL relation, was centrifuged for 5 min at 5,000 × g at 22°C ± 1°C using a Sorvall ST 16R centrifuge (Thermo Fisher Scientific). The pellets were suspended in 50 mL of raw bovine milk to obtain a final concentration of approximately 8 log_{10} cfu/mL. Three different milks were used and were contaminated with independent STEC cultures. Suspensions were 10-fold serially diluted in raw milk to obtain four 45-mL samples of contaminated raw bovine milk for each strain. The samples contained approximately 8 log_{10}, 7 log_{10}, 6 log_{10}, or 5 log_{10} cfu/mL. We collected three 100-µL aliquots from each *E. coli*-contaminated milk, serially diluted the aliquots in tryptone salt broth, and immediately plated them in duplicate on *E. coli* Brilliance medium (ECB, Thermo Fisher). We used a Whitley Automated Spiral Plater (AES Chemunex, BioMérieux) to enumerate *E. coli*. Plates were incubated for 18 to 24 h at 37°C, and colonies were counted. Raw milk suspensions were placed at 4°C for 16 to 24 h, to allow natural cream separation (creaming). After creaming, the volume of the cream layer was evaluated by reading directly on the tube (Falcon tube, 50 mL, Corning Life Sciences). We col-
selected three 100-µL samples from the cream layer in each tube, taking care to sample different areas. *Escherichia coli* were enumerated as previously described for raw milk.

In addition, total microflora, mesophilic coliforms, thermotolerant coliforms, *Enterobacteria*, and *E. coli* of raw milk used were counted. Briefly, raw milk was serially diluted in tryptone salt medium (BioMérieux), plated twice on specific media, and incubated according to manufacturer recommendations (Table 2).

**Estimation of SC by Nonlinear Regression.** For each tube, the concentration of *E. coli* in milk (*C* _milk_ in cfu/mL) and in cream (*C* _cream_ in cfu/mL) was estimated by the sum of the 3 bacterial counts divided by the total volume of the 3 samples. We hypothesized that if STEC strains have an affinity for MFG in milk, STEC cells would be concentrated in the cream layer. The concentration of STEC in the cream should thus be dependent on the concentration factor (*a*). The concentration of STEC did not affect the volume of cream (*V* _cream_ in mL); therefore, for each tube, *a* is only dependent on the milk and is equal to the ratio of the volume of milk (*V* _milk_ in mL) to the volume of cream (*V* _cream_ in mL).

Thus, if our hypothesis is true, STEC cells are all concentrated in the cream. If a saturation phenomenon exists, we expect this relation to be true only for low *C* _milk_ values, whereas *C* _cream_ would reach a saturation (*C* _max_ ) at high *C* _milk_ from a concentration threshold, which we called the SC. The SC may depend on the STEC strain. We estimated the SC for each STEC strain from observed data, assuming SC does not depend on the milk. For this purpose, we fitted the following simple model for each STEC strain to the *C* _cream_ values obtained:

\[
C_{\text{cream}_j} = C_{\text{milk}_j} \times a_j \quad \text{if} \quad C_{\text{milk}_j} < SC,
\]

and

\[
C_{\text{cream}_j} = SC \times a_j \quad \text{if} \quad C_{\text{milk}_j} \geq SC,
\]

with *i* the index for milk serial dilutions (*i* = 1 to 4), and *j* the index for milks (*j* = 1 to 3). In this model, only the threshold parameter SC is estimated for each strain, the values of *a* being calculated as *V* _milk_/*V* _cream_ for each milk *j*. We estimated SC by nonlinear regression using the nls() function of R software (R Core Team, 2021) after logarithmic transformation of the concentrations to respect the hypothesis of the Gaussian error model:

\[
\log_{10} \left( C_{\text{cream}_j} \right) = \log_{10} \left( C_{\text{milk}_j} \right) + \log_{10} \left( a_j \right) \quad \text{if} \quad C_{\text{milk}_j} < SC,
\]

and

\[
\log_{10} \left( C_{\text{cream}_j} \right) = \log_{10} \left( SC \right) + \log_{10} \left( a_j \right) \quad \text{if} \quad C_{\text{milk}_j} \geq SC.
\]

**E. coli Saturation Test in Bovine Raw Milk Cream by Sucrose Density Gradient Centrifugation Assay**

**Experimental Plan.** A diagram describing the experimental plan design is available in the supplemental data (Supplemental Figure S2; http://dx.doi.org/10.17605/OSF.IO/K86TA; Bagel et al., 2022). Three bovine raw milk samples were spiked to 10 log10 cfu/mL with each selected STEC strain, as previously described in the natural creaming assays. The strains O157:H7 str. EDL933 and O26:H11 str. 21765 were selected as model strains due to their different affinities for cream (these strains showed the highest and lowest SC). All STEC-spiked raw milk suspensions were 10-fold serially diluted with raw milk to obtain a range of contaminated milk concentrations for each strain and were incubated at 4°C for 30 min to allow STEC-MFG adhesion. All STEC-contaminated raw milks were 10-fold serially diluted in tryptone salt and plated in duplicate on ECB medium (18–24 h at 37°C) to enumerate STEC. Then, to separate STEC cells associated with MFG from those that were not, a sucrose density gradient (SDG) centrifugation assay was used as described in Brisson et al. (2010). Briefly, 4 mL of each *E. coli*-contaminated milk was added to the top of a double phase system consisting of 5 mL of 20% sucrose and 5 mL of 60% sucrose (Sigma Aldrich, Merck). Samples were then centrifuged at 4,000 × *g* for 30 min at 4°C. Both MFG

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**Table 2. Media and growth conditions used for raw milk microbiological analysis**

| Microbiological class | Agar plate                                      | Growth conditions   |
|-----------------------|------------------------------------------------|---------------------|
| Total mesophilic flora | Plate count agar (PCA, Oxoid) + 0.1% skim milk (Regilait) | 30°C; 72 h          |
| *Enterobacteria*      | Violet red bile glucose agar (VRBG, Biokar)    | 37°C; 24 h          |
| Mesophilic coliforms  | Violet red bile lactose agar (VRBL, Biokar)    | 30°C; 24 h          |
| Thermotolerant coliforms | Violet red bile lactose agar (VRBL, Biokar) | 44°C; 24 h          |
| *Escherichia coli*, α-glucuronidase positive | Tryptone bile X-glucuronide medium (TBX, Oxoid) | 44°C; 24 h          |
| *Escherichia coli* and coliforms | Brilliance *E. coli*/Coliform-selective agar (ECB, Oxoid) | 37°C; 24 h          |
and STEC-MFG complexes have a lower density than sucrose solutions and, therefore, rise to the surface with a minimal amount of raw milk proteins, whereas STEC not associated with MFG are pelleted. The MFG and STEC-MFG complexes were recovered with a sterile spatula and dispersed in 4 mL of sterile PBS (Sigma Aldrich, Merck). Creams were enumerated for STEC as previously described for raw milk.

**Estimation of SC by Nonlinear Regression.** For each condition, we disposed of the measured concentration of STEC in milk ($C_{\text{milk}}$, in cfu/mL) and in cream ($C_{\text{cream}}$, cfu/mL). As previously described, we fitted the following simple model for each STEC strain to values of $C_{\text{cream}}$ obtained:

$$C_{\text{cream}}_i = C_{\text{milk}}_i \times a \text{ if } C_{\text{milk}}_i < SC,$$

and

$$C_{\text{cream}}_i = SC \times a \text{ if } C_{\text{milk}}_i \geq SC,$$

with $i$ the index for milk serial dilutions ($i = 1$ to 4). In this experiment, the volume of cream was resuspended in the same volume of milk; thus, $a = 1$ for each milk.

**Evaluation of Cream Physical Congestion by Competitive E. coli Saturation Test**

Competitive natural creaming of raw milk was performed with 2 STEC: O157:H7 str. EDL933 and O26: H11 str. 21765. Strains were selected according to their affinity for the cream layer (high and low SC) and the colony phenotype on ECB medium. In our collection, only O157:H7 strains are β-glucuronidase negative (GUD−) and cannot cleave the chromogenic substrate X-Glu added to the ECB medium. Strains belonging to O26:H11 or O103:H2 serotypes appear as purple colonies (LAC+, GUD+), whereas O157:H7 strains appear pink (LAC+, GUD−) on ECB agar plates. Natural creaming assays were carried out following the protocol described in the previous section, with several modifications. Raw milk was experimentally spiked to 8 log_{10} cfu/mL with O157:H7 str. EDL933 and O26:H11 str. 21765, and placed at 4°C for 18 to 24 h. Raw milk was also spiked to 8 log_{10} cfu/mL with each strain for the control experiment (no competition). Creaming with no enrichment step (no STEC) was also performed, to estimate the natural microflora in the cream layer. Purple and pink colonies were enumerated from cream and raw milk and were identified as the 21765 and EDL933 strains, respectively. The serogroup of the colonies was verified by agglutination tests (SlideX E. coli O26, BioMérieux; E. coli O157 Latex, Oxoid, Thermo Fisher Diagnostics). Dual- and single-strain creaming assays were performed in triplicate (3 independent cultures).

**Effect of Fat Content on STEC Concentration in Cream**

To confirm the role of milk fat in the phenomenon of saturation, we performed an SDG centrifugation assay as previously described but with diluted raw milk. Raw milk was diluted 2-fold or 10-fold with UHT skimmed bovine milk and experimentally spiked to 6 log_{10} cfu/mL with O157:H7 str. EDL933 or O26:H11 str. 21765.

**Evaluation of Adhesion Capacity of Non-Adherent STEC Cells**

To confirm the phenotype of STEC cells that did not associate with MFG, pelleted STEC cells were used to spike uncontaminated raw milk, and the SDG centrifugation test was performed several times. Raw milk was spiked with the O157:H7 str. EDL933 to a final concentration of 6 log_{10} cfu/mL and incubated at 4°C for 30 min. Then, 10 mL of spiked milk was added to the top of a double-phase system consisting of 15 mL of 20% sucrose and 15 mL of 60% sucrose in a 50-mL tube. Samples were then centrifuged at 4,000 × g for 30 min at 4°C, as previously described. Milk fat globules were recovered with a sterile spatula and dispersed in 10 mL of sterile PBS. Sucrose solutions were gently removed with a vacuum pump. Pellets containing unassociated bacteria were transferred to a new tube, washed with 5 mL of sterile PBS, and pelleted again by centrifugation for 5 min at 5,000 × g at 4°C. Washed pellets were used to spike 10 mL of uncontaminated bovine raw milk and were treated as previously described. In total, 3 cycles of contamination – centrifugation – pellet isolation – contamination were performed. Both MFG and spiked raw milk were 10-fold serially diluted in tryptone salt, plated in duplicate on ECB medium, and incubated for 18 to 24 h at 37°C to enumerate STEC. The concentration of STEC in the pellet was determined by enumeration of STEC in the newly contaminated raw milk. Each experiment was repeated to ensure reproducibility (i.e., 2 different cultures were performed).

**Microscopic Observations**

**Confocal Laser Scanning Microscopy.** Confocal laser scanning microscopy was used to localize E. coli cells in bovine raw cream. With a Nikon A1R microscope (Nikon) installed in a non-biosafety level 3 laboratory, the model strain AEEC O26:H11 was used for CLSM observations, and the experiments were realized in a BSL-2 laboratory. Raw bovine milk was inoculated
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RESULTS

Affinity and SC of E. coli Strains in Bovine Raw Cream

To evaluate the affinity of STEC and AEEC strains for bovine raw MFG, natural creaming saturation tests were performed for a set of 16 E. coli strains (Table 1). Figure 1 shows the concentration of each E. coli strain in cream as a function of the initial concentration in raw milk with the regression curve for each milk (n = 3). All data are available in Supplemental Figure S3 (http://dx.doi.org/10.17605/OSF.IO/K86TA; Bagel et al., 2022). Table 3 shows the bacterial count of each raw milk used in this experiment.

The concentration of each E. coli strain in the cream obtained by natural creaming was ~1 log10 cfu/mL greater than the initial enrichment level (Figure 1). Escherichia coli strains were mainly recovered in the cream layer when the bacterial level in raw milk ranged from 5 to 7 log10 cfu/mL or from 5 to 8 log10 cfu/mL, depending on the strain (Figure 1). However, at higher inoculum levels—7 or 8 log10 cfu/mL—the concentration of E. coli in the cream was limited; the cream layer appeared saturated by E. coli strains (Table 1).

Transmission Electron Microscopy. The MFG were washed to remove unadsorbed proteins from the samples, using a protocol adapted from (Patton and Huston, 1986). Briefly, raw milk samples were warmed to 40°C, and then 10 mL of milk was mixed with 5 mL of a 0.22-µm-filtered 50% (wt) sucrose solution. The milk/sucrose sample was deposited at the bottom of a 50-mL centrifuge tube filled with 30 mL of a 5% (wt) sucrose solution. Centrifugation was performed for 20 min at 22°C ± 1°C to form a layer of washed MFG at the top of the tubes. The top layer, containing the washed MFG, was collected in an adequate volume of phosphate buffer (43 mM NaH2PO4/Na2HPO4; pH = 6.8; ionic strength = 80 mM) to approximate the pH and ionic strength of milk. Sucrose, NaH2PO4, and Na2HPO4 were supplied by Sigma Aldrich (Merck).

Purified MFG were contaminated with approximately 8 log10 cfu/mL of a stationary STEC or AEEC culture (BHI, 37°C) and kept for 2 h at 19°C ± 1 to allow association. Contaminated creams were then labeled with 2 mg/mL cationized ferritin (Electron Microscopy Sciences, Delta Microscopies) for 30 min at 19°C ± 1. Samples were then mixed with 3% low-temperature gelling agarose solution (Electron Microscopy Sciences) maintained at 40°C (vol/vol). One hundred microliters was immediately placed into a disposable plastic 1-mm-thick mold. Solidified samples were cut into 1-mm3 blocks and then immersed in a 2% glutaraldehyde solution (0.2 M; cacodylate buffer; pH 7.2; Electron Microscopy Sciences) at 4°C. All pieces were rinsed 3 times in cacodylate-Na-HCl, pH 7.4, 0.2 M (Electron Microscopy Sciences) for 1 h at 4°C and post-fixed with 2% OsO4 in cacodylate-Na-HCl, pH 7.4, 0.3 M (Electron Microscopy Sciences) for 1 h at 4°C. Afterward, samples were dehydrated in a graded series of ethanol and transferred to propylene oxide. Impregnation was performed with Epon epoxy resin (Electron Microscopy Sciences). Inclusion was obtained by polymerization at 60°C for 72 h. Ultrathin sections (approximately 70 nm thick) were cut on a UC7 (Leica) ultramicrotome, mounted on 200-mesh copper grids coated with 1:1,000 poly-L-lysine, stabilized for 1 d at room temperature, and contrasted with uranyl acetate and lead citrate. Sections were examined with a 1400JEM 120 kV transmission electron microscope (Jeol).

RESULTS

Affinity and SC of E. coli Strains in Bovine Raw Cream

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8.21 log_{10} cfu/mL with a 95% confidence interval (CI) [7.98; 8.45]. The fitting procedure failed to estimate the CI of the O103:H2 str. PMK5, due to the lack of observed points in the saturation phase. An SC greater than the maximal contamination level (C_{max}; represented as black bars in Figure 2) means that the saturation point has not been reached. This was the case for 4 strains: PMK5, which had an undefined upper CI; the O157:H7 2044-A and 1044 strains; and the nonpathogenic E. coli K-12 str. MG1655 (Figures 1

Figure 1. Saturation curves of Escherichia coli strains in raw milk cream obtained by natural creaming assay. The concentration of each E. coli strain in the raw milk cream layer as a function of its initial concentration in milk was plotted for each milk used (points) as well as the regression curve (flat line). Colors represent different milks (n = 3).
The volume of cream obtained at the end of the natural creaming experiments and the calculated cream concentration factor, a parameter of the nonlinear model used for estimated SC, are presented in Table 4.

All strains belonging to the O26:H11 serotype saturated the cream layer and could be parsed into 2 groups based on the SC value. The cream layers were saturated at either ~7 log_{10} cfu/mL (4315-A and 21765 strains; group 1) or ~7.5 log_{10} cfu/mL (11368, 103, and 12157-A strains, group 2). However, both O26:H11 strain groups showed lower SC than the O157:H7 strains. The strains belonging to the O157:H7 serotype presented a very low intravariability compared with the other serotypes and displayed an SC at ~8 log_{10} cfu/mL. For the O103:H2 serotype, only the human-pathogenic PMK5 strain presented a distinct profile. The cream layer was not fully saturated in these conditions (Figure 1), and the model could not estimate the SC (Figure 2). The other O103:H2 strains displayed a similar SC at ~7.5 log_{10} cfu/mL.

The STEC associated with MFG were purified from raw milk using an SDG centrifugation test. The experiment was performed with 2 pathogenic STEC strains that showed extreme SC values: O157:H7 str. EDL933 and O26:H11 str. 21765. The majority of viable STEC cells were found in the cream fraction up to a concentration threshold (Figure 3A) as previously described for natural creaming experiments. The SC of the O26: H11 str. 21765 was estimated at 5.98 log_{10} cfu/mL, whereas O157:H7 str. ELD933 saturated the cream at 7.22 log_{10} cfu/mL (Figure 3B).

Figure 2. Estimation of the saturation concentration (SC) of Escherichia coli strains in the raw cream layer from natural creaming assay. Segments represent 95% CI. For the E. coli O103:H2 str. PMK5 strain, the model could not estimate CI. Black bars represent the mean of the maximal spiked level (C_{milk,max}). Strains with SC above this threshold were considered nonsaturating under these conditions. The genotype and the origin of the strains are indicated in different colors.
Localization of the O26:H11 AEEC Strain Around MFG

In the CLSM images, the E. coli cells specifically labeled with FITC anti-O26 appeared as green cells (Figure 4A-1). As expected, MFG were concentrated in the cream. Our CLSM observations showed that the O26:H11 AEEC cells were located around some, but not all, MFG (Figure 4A). The AEEC cells were observed in close contact with an MFG (Figure 4A-5). In addition, some AEEC cells were visualized in the serum phase of the cream.

Using transmission electron microscopy, AEEC cells were observed in contact with the MFGM for each strain and serotype assayed (Figure 4B). For the strains belonging to the O157:H7 and O26:H11 serotypes, the largest side of bacterial cells was in contact with the MFGM. Pili were observed for some strains (Figure 4B-2, B-3), but no large extracellular appendages were visualized in contact with the MFGM.

Table 4. Volume of the cream layer (V_{cream}) obtained by natural creaming assay and the log_{10} of the concentration factor (a) for each strain and repetition

| Serotype and strain | V_{cream} (mL); log_{10}(a) |
|---------------------|-----------------------------|
|                     | Repetition 1 | Repetition 2 | Repetition 3 |
| O26:H11             |              |              |              |
| 103                 | 5; 0.95      | 5; 0.95      | 7.5; 0.78    |
| 11368               | 5; 0.95      | 5; 0.95      | 7.5; 0.78    |
| 2157-A              | 5; 0.95      | 5; 0.95      | 7.5; 0.78    |
| 21765               | 5; 0.95      | 5; 0.95      | 7.5; 0.78    |
| 4315-A              | 5; 0.95      | 5; 0.95      | 7.5; 0.78    |
| O157:H7             |              |              |              |
| 1044                | 5; 0.95      | 8; 0.75      | 6; 0.88      |
| 2044-A              | 5; 0.95      | 8; 0.75      | 6; 0.88      |
| 5280-A              | 5; 0.95      | 8; 0.75      | 6; 0.88      |
| EDL933              | 5; 0.95      | 8; 0.75      | 6; 0.88      |
| SAKAI               | 5; 0.95      | 8; 0.75      | 6; 0.88      |
| O103:H2             |              |              |              |
| 1487-A              | 5; 0.95      | 7.5; 0.78    | 7.5; 0.78    |
| 2503                | 5; 0.95      | 7.5; 0.78    | 7.5; 0.78    |
| 32396               | 5; 0.95      | 7.5; 0.78    | 7.5; 0.78    |
| 445-14              | 5; 0.95      | 7.5; 0.78    | 7.5; 0.78    |
| PMK5                | 5; 0.95      | 7.5; 0.78    | 7.5; 0.78    |
| K-12 MG1655         | 5; 0.95      | 5; 0.95      | 5; 0.95      |

Figure 3. Shiga toxin-producing *Escherichia coli* cream saturation assay obtained by sucrose density gradient centrifugation test. (A) Saturation curve of O157:H7 str. EDL933 and O26:H11 str. 21765 for raw milk cream. For each strain, the concentration in cream versus the initial concentration in milk was plotted (point), as well as the regression curve (flat line). (B) Estimation of the saturation concentration by nonlinear regression (filled circle). Segments represent 95% CI.
Spatial Congestion of E. coli Cells in Cream Evaluated by a Competitive Natural Creaming Assay

To evaluate whether bacterial spatial congestion could be responsible for the saturation of STEC in cream, we performed a competitive natural creaming assay, by experimentally spiking milk with 2 STEC strains with significantly different SC. The concentration of O157:H7 str. EDL933 in cream was 0.50 log_{10} and 0.45 log_{10} cfu/mL higher than the initial concentration in raw milk for the control and competition tests, respectively (Figure 5). The difference in bacterial concentration in cream was less than −0.08 log_{10} cfu/mL for O26:H11 str. 21765 in both conditions (Figure 5). No difference was observed between the control (single strain) and competition (2 strains) samples. These data suggest that the O157:H7 str. EDL933 strain could still be concentrated in an already saturated cream.

Effect of Raw Milk Fat Content on STEC Concentration in Cream

The initial STEC concentration in raw milk was fixed to 6 log_{10} cfu/mL. The STEC concentration in cream decreased as a function of fat content (Figure 6). The STEC concentration in cream from undiluted raw milk was 5.43 log_{10} cfu/mL for O26:H11 str. 21765 and 5.89 log_{10} cfu/mL for O157:H7 str. EDL933. With EDL993, a majority of STEC cells were recovered from the cream layer of raw milk, whereas for 21765, only half of the cells were recovered from the cream layer. In half-diluted raw milk, the concentrations were 4.94 log_{10} cfu/mL for O26:H11 str. 21765 and 5.69 log_{10} cfu/mL for O157:H7 str. EDL933. A greater difference in STEC concentration in cream was found when the raw milk was diluted to 1:10 to 3.63 log_{10} cfu/mL for O26:H11 str. 21765 and 4.57 log_{10} cfu/mL for O157:H7 str. EDL933. Therefore, although the trend was similar, an obvious strain effect occurred. We recovered less of the O26 strain than the O157 strain from cream for each dilution.

Characterization of STEC Cells That Do Not Associate With Raw Milk Cream

We also studied those STEC cells that did not localize in cream. The median of STEC concentration in cream was 6.5 log_{10} cfu/mL, and the median concentration in the pelleted fraction was estimated to be 5.10 log_{10} cfu/mL (Figure 7A). The pellet was used to contaminate new raw milk samples to study why these cells did not localize in the cream. We found no difference in the concentrations of pelleted STEC cells between the first and the second cycle: ~1.5 log_{10} cfu/mL less than the raw milk concentration for both cycles (Figure 7B). The majority of STEC cells were recovered in the cream for the first 2 cycles (Figure 7B). In the last purification-contamination cycle, more STEC cells were also recovered from the cream layer: 3.38 log_{10} cfu/mL in the cream layer and 2.54 log_{10} cfu/mL in the pellet (Figure 7A). However, the concentration of pelleted STEC cells was higher in the third cycle (~0.5 log_{10} cfu/mL) than previous cycles (Figure 7B).

DISCUSSION

Historically, creaming was used to concentrate the fat of raw milk and to purify it from bacteria. During creaming, MFG spontaneously rise to the surface because they are less dense than the aqueous phase, forming an MFG-concentrated layer known as cream (Mulder and Walstra, 1974; Euber and Brunner, 1984; Bird, 1991). These observations suggest that the natural microbiota of raw milk have a certain affinity for raw milk fat. Unfortunately, pathogenic bacterial cells can contaminate raw milk, leading to human infection through ingestion of contaminated raw milk products. Shiga toxin-producing E. coli is a true challenge for the dairy industry, and better understanding of the behavior of this pathogen in dairy products is important to identify new preventive strategies. Therefore, in this study, we aimed to evaluate the affinity of STEC for raw milk cream, to better understand the concentration of this pathogen in cream and to discover whether this concentration varies according to serotype.

We used natural creaming assays to analyze the affinity of 12 STEC strains, 3 AEEC strains, and one non-pathogenic E. coli strain (K-12 str. MG1655) for raw milk cream (Figure 1). Our data showed that STEC cells were mainly recovered from cream. Approximately 1 log_{10} cfu/mL more STEC was observed in the cream layer than in raw milk after natural creaming, indicating a 10-fold increase in STEC concentration in cream, compared with raw milk. Creaming was performed at a low temperature to limit bacterial growth. No STEC growth was observed in spiked raw milk after 24 h at 4°C (data not shown). Previous studies have shown that natural creaming can concentrate up to 500 times more bacteria in cream than in milk (Anderson, 1909; Lamson, 1918), which is significantly higher than the STEC concentration levels obtained in this study. Furthermore, in this study, the distribution of all strains in the cream was homogeneous (data not shown). Of note, E. coli K-12 str. MG1655, as well as the AEEC
strains, were also concentrated in the cream, suggesting that the affinity for raw milk cream is not specific to the STEC pathotype but could be more general to *E. coli*. Moreover, the concentration of STEC in the cream seemed to be fat level dependent (Figure 6).

In this work, we also aimed to quantify the phenomenon of cream saturation by STEC, as suggested in a previous study (Douéllou et al., 2018). The STEC cells were not fully concentrated in the cream at high concentrations (>7 log_{10} cfu/mL; Figure 1), especially for the O103:H2 and O26:H11 strains. To compare the strains, we estimated the SC by nonlinear regression, defined as the lowest STEC concentration in raw milk for which bacteria were no longer concentrated in the cream layer. The SC (the concentration threshold in milk) was selected rather than the parameter $C_{\text{max}}$ (the maximal concentration of STEC in the cream layer) because raw milk product contamination by STEC is mainly due to milk contamination at the farm level. The parameter $C_{\text{max}}$ was also estimated (data not shown), and the conclusions of our study did not change (correlation of both parameters). Furthermore, as $C_{\text{milk}}$ was a control parameter in our experiment, this parameter was used as a threshold to establish whether the cream layers were saturated or not (Figure 2). In addition, the volume of cream was always the same, regardless of STEC levels; however, some differences were observed between different milks (Table 2).

Figure 4. Microscopy observations of Shiga toxin-producing *Escherichia coli* (STEC) and attaching-effacing *Escherichia coli* (AEEC) strains in raw milk cream. (A) Confocal laser scanning microscopy observations of O26 AEEC cells specifically labeled with fluorescein-5-isothiocyanate anti-O26 in PBS (A-1) and in the cream obtained by natural creaming from raw milk previously enriched with the O26 AEEC strain at 5 log_{10} cfu/mL (A-2 to A-5). The triglyceride core of MFG appears in red and AEEC cells in green. (B) Transmission electron microscopy (TEM) images of several STEC or AEEC strains in washed cream containing milk fat globules enveloped by their natural biological membrane. In all the TEM images, the scale bars correspond to 0.5 µm.
Based on SC values, the strains clustered into 3 groups: (1) ~7 log_{10} cfu/mL; (2) ~7.5 log_{10} cfu/mL; and (3) > 7.8 log_{10} cfu/mL (Figure 2). No correlation, other than serotype or strain, was found that could explain these clusters (e.g., origin, stx gene). A genome-wide study including 197 strains showed similar strain clustering based on serotype (Douëllou et al., 2017a). Another study showed the O26: H11 str. 11368 (tested in our study) was in the same cluster as an E. coli strain belonging to the O103:H2 serotype (Wyrsch et al., 2015). More E. coli strains, especially eae−, as well as other enterobacteria, should be assayed to extend and explain these findings.

Our data showed that all E. coli O157:H7 strains were located in the same cluster. The estimated SC of O157:H7 strains were between 7.86 log_{10} and 8.21 log_{10} cfu/mL (Figure 2), which was approximately equivalent to the inoculation concentration (~8 log_{10} cfu/mL). Thus, cream saturation was not fully achieved under the tested conditions for these strains and should probably be higher. In contrast, all strains belonging to the O26: H11 serotype reached saturation. Only one O103:H2 strain (str. PMK5) did not saturate cream in these conditions, whereas the other strains exhibited similar SC at ~7.5 log_{10} cfu/mL (Figure 2). These data are in agreement with previous saturation tests conducted in the laboratory on O26:H11 str. 21765 and O157:H7 str. EDL933 (Douëllou et al., 2018). The behavior of E. coli K-12 str. MG1655 was very similar to the STEC O157 strains, displaying a high saturation point. The SC of O103:H2 str. PMK5, O157:H7 str. 2044-A and str. 1044, and the nonpathogenic E. coli str. K-12 MG1655 were not reached in our experimental conditions (Figure 1; Figure 2). These results suggest that the saturation point of these strains is probably higher than the maximum initial concentration of E. coli studied in this experiment. For O157:H7 str. EDL933, a wider range of contamination level (3 log_{10} cfu/mL to 10 log_{10} cfu/mL) was tested by SDG centrifugation assay, and, under these conditions, the cream was saturated at 7.22 log_{10} cfu/mL (Figure 3A).

Furthermore, AEEC strains exhibited SC close to those of STEC strains belonging to the same serotype. Thus, STEC and AEEC seem to have similar affinities for raw milk fat. In addition, all assayed strains displayed a high SC (6.94 to 8.05 log_{10} cfu/mL), compared with the levels observed in products (<10 cells; (Farrokh et al., 2013)). These data suggest that STEC cells should be mainly found in the cream of naturally contaminated raw milk. However, it is very likely that some cells are still present in the serum phase of the milk. Therefore, further experiments with low STEC contamination levels should be performed.

Confocal microscopy observations of AEEC-contaminated raw milk cream layers showed that MFG could have a pivotal role in the phenomenon of concentra-
tion, as bacterial cells were localized near MFG (Figure 4A). The affinity of bacteria for MFG has been widely demonstrated (Ly et al., 2006; Brisson et al., 2010; Ly-Chatain et al., 2010; D’Incecco et al., 2015; Gomand et al., 2018; Guerin et al., 2018). In this study, our data confirmed that STEC, as well as AEEC, could associate with MFG.

Based on these observations, we then assessed whether the saturation was due to bacterial spatial congestion rather than to a specific affinity and whether STEC found in the cream were associated with MFG. We also evaluated whether the STEC cells recovered from the milk layer were unable to associate with the cream or MFG.

First, we hypothesized that the presence of additional STEC in the cream is physically not possible, and, as a result, a strain with a high SC can still concentrate in the cream when another strain is already saturating the cream. Competitive natural creaming assays showed that a STEC strain with high cream saturation potential was mainly concentrated in the cream even though the cream was already saturated by a STEC strain with a lower SC (Figure 5). Therefore, the mechanism of STEC concentration in cream may be strain specific.

Second, at low temperatures, MFG rapidly aggregate to form a network (Euber and Brunner, 1984). Some bacterial cells may be trapped in this network and rise with the MFG, to be concentrated in the cream. The SC could, therefore, be overestimated by the natural creaming method. We used the SDG centrifugation test to confirm that STEC recovered in cream were associated with MFG and not simply trapped by them. The results of the SDG centrifugation tests showed the same pattern as natural creaming: STEC concentration in cream at high levels (approx. 6 log10 or 7 log10 cfu/mL, depending on the strain) and a saturation of the cream (Figure 3A). For 2 model strains, STEC-raw milk fat affinity profiles obtained by natural creaming were confirmed by SDG centrifugation assay. These data strongly suggest that the STEC recovered in cream were associated with MFG.

However, the SC determined by SDG centrifugation tests were lower than the SC estimated with the natural creaming method for both strains. We observed approximately 0.96 log10 cfu/mL difference for the O26 strain and 0.68 log10 cfu/mL difference for the O157 strain (Figure 2; Figure 3B). Furthermore, we found higher variability of the SC with SDG centrifugation tests for both strains, as shown by the larger CI (approximately 1 log10 cfu/mL; Figure 2; Figure 3B). In the SDG assays, the MFG were recovered manually, and a portion of them may have been lost, which could explain both the variability (different quantity recovered per repetition) and the lower estimated SC with this method. In addition, the high sucrose content could affect the STEC-MFG association. However, regardless of the fat separation method, the SC of the O26 strain was always lower than the SC of the O157 strain.
It is important to point out that, although the 2 separation methods provided different SC, these values remain of the same order of magnitude and specific to each strain. The method-specific differences in SC suggest that several mechanisms may be involved in STEC concentration in cream, such as adhesion of STEC to MFGM components, trapping of STEC by MFG, or involvement of other components of raw milk. Ma and Barbano (2000) found that milk with a high bacterial content had a lower fat content (approximately 50–55%) after creaming than milk with a low bacterial content (approximately 80%). In addition, bacteria seem to act as a physical aggregation barrier, and high bacterial content can disturb flocculation and, consequently, raw milk creaming capacity (Dos Santos Morais et al., 2021).

Brewster and Paul (2016) showed that the cream layer exhibits a high containment capacity for Escherichia coli O157:H7, Listeria monocytogenes, and Salmonella enterica. Half-saturation for raw milk cream was estimated at approximately 8.5 log_{10} cfu/mL. The same study revealed that the majority of pathogenic bacterial cells added to pasteurized or homogenized milk were recovered in the pellet after simple centrifugation of milk. Heat treatment greatly affects the proteins concentrated in the cream layer. Moreover, the literature shows that the microflora can affect the viability of some bacterial species, and therefore their detection. Hafnia alvei has been shown to inhibit STEC O26:H11 in raw milk cheese (Delbès-Paus et al., 2012). Starter lactic acid bacteria, molds, as well as intrinsic microflora may act as competitors against STEC O157:H7 (Duffy et al., 1999; Rios et al., 2020). Although some evidence suggests that raw milk microbiota may contain antagonistic bacteria against STEC, the saturation phenomenon observed in this study is real, as it was observed in the SDG experiment. Indeed, in this experiment, STEC were not in contact with the potential antagonistic microflora of raw milk long enough to observe an effect. We would also emphasize that the variability of the bacterial counts of the cream layer, between the different milks used, is low, underlining that the effect of the microflora is minimal. Our study was not intended to demonstrate competition between microflora and STEC for MFG. It would be interesting to further study the influence of raw milk microflora on the affinity of STEC for MFG.

The results of our study implicate MFG in the phenomenon of cream saturation and show that, during natural creaming, STEC can be in contact with MFG, probably through specific interactions. The association of STEC with MFG could involve sugars (glycoproteins and glycolipids) anchored in the MFGM (Douéllou et al., 2017b; Guerin et al., 2018; Bagel and Sergentet, 2022). No large bacterial structures were observed in direct contact with the MFGM. Furthermore, no flagella were observed. Nevertheless, for certain strains, some surface structures were observed (Figure 4B). Shiga toxin-producing E. coli have a collection of specific adhesins on their surface (McWilliams and Torres, 2014) that recognize sugar moieties of MFGM glycoproteins or glycolipids. Also, somatic cells and immunoglobulins present in raw milk seem to be involved in the concentration of bacteria in the cream layer (Ma and Barbano, 2000). The results of our study implicate MFG in the phenomenon of cream saturation and show that, during natural creaming, STEC can be in contact with MFG, probably through specific interactions. The association of STEC with MFG could involve sugars (glycoproteins and glycolipids) anchored in the MFGM (Douéllou et al., 2017b; Guerin et al., 2018; Bagel and Sergentet, 2022). No large bacterial structures were observed in direct contact with the MFGM. Furthermore, no flagella were observed. Nevertheless, for certain strains, some surface structures were observed (Figure 4B). Shiga toxin-producing E. coli have a collection of specific adhesins on their surface (McWilliams and Torres, 2014) that recognize sugar moieties of MFGM glycoproteins or glycolipids. Also, somatic cells and immunoglobulins present in raw milk seem to be involved in the concentration of bacteria in the cream layer (Ma and Barbano, 2000).
Barbano, 2000; Caplan et al., 2013; Geer and Barbano, 2014).

From a quality and product safety point of view, our results do not allow us to advise dairy industries on new measures to take. Further studies more specific to current industrial practices are needed. However, our results show that STEC affinity for bovine raw milk or MFG is strain and serotype specific. The underlying mechanism could involve specific interactions between STEC and MFG. The affinity of STEC for MFG and, in particular, the molecular interactions that are involved, need to be further investigated. The identification of molecular targets could lead to new prevention strategies.

CONCLUSIONS

This study demonstrated the affinity of STEC and AEEC strains for raw milk cream and MFG. This affinity was found to be strain and serotype dependent. The results also showed that the affinity and saturation of the cream layer or MFG are not specific to STEC or AEEC strains but may be more generalized to E. coli. According to the results of our study, other pathogens or microorganisms could concentrate in the cream layer. Thus, our study could be used as an adhesion or a saturation model for other pathogens, spoilers, and microorganisms in raw milk and the raw milk cream layer. The SC could vary according to the strains. However, STEC were not fully concentrated in the cream layer; therefore a microbiologic hazard remains present in raw milk regardless of the enrichment level, especially for STEC, which has a very low infectious dose. Therefore, natural creaming should not be used as preventive tool to eliminate STEC from bovine raw milk. A more detailed study should be conducted, with industrial creaming practices and STEC spiking conditions closer to field reality, to confirm and extend these conclusions. In addition, the mechanisms of interaction between STEC and dairy product components should be investigated to highlight new tools to control STEC infection and develop therapeutic antiadhesion strategies.

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