Tenacity of Enterococcus cecorum at different environmental conditions

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Keywords
broiler, enterococcal spondylitis, Enterococcus cecorum, humidity, litter, PVC, survival, temperature.

Abstract
Aims: Our aim was to analyse the survival of Enterococcus cecorum (EC) at various temperatures, relative air humidities and on different substrates commonly existing in broiler houses.

Methods and Results: A pathogenic EC isolate (EC14) was used to inoculate sterile litter, polyvinyl chloride (PVC) and dust samples. Incubation at 37, 25 or 15°C with either 32% relative humidity (RH) or 78% RH followed. At defined time points (0–4272 h post-inoculation), samples were examined in triplicate for the total viable count. Selected combinations were repeated for a non-pathogenic and two additional pathogenic EC strains. For EC14, the measured survival time ranged from 48 to 4272 h (178 days) depending on the substrate–humidity–temperature combination. The longevity was the highest on litter, followed by dust and then PVC. Lower temperatures facilitated its survival, lower relative air humidity favoured the survival only in combination with 25 or 15°C. All three pathogenic strains showed longer survival times (up to 432 h, 18 days) compared to the non-pathogenic EC strain (168 h, 7 days) under the same conditions.

Conclusions: Enterococcus cecorum demonstrates a high persistence in the environment especially at 15°C and 32% RH.

Significance and Impact of the Study: Hygiene management plans should consider the durability of EC and the risk of a carry-over to control consecutive EC outbreaks.

Introduction
Enterococcus cecorum (EC) is a Gram-positive bacterium, which is facultative anaerobic and a non-spore former. It can be isolated from many animals as well as from humans (Devriese et al. 1991a; Delaunay et al. 2015; Jung et al. 2017b), and it is known to be a commensal of the gut, especially of chickens (Devriese et al. 1991a, b). However, since 2002, disease outbreaks caused by EC have been reported in broiler chickens (Devriese et al. 2002; Wood et al. 2002), and it is considered an emerging avian pathogen today (Jung et al. 2017a). The affected chickens show lameness or hock sitting due to spondylitis and osteomyelitis in the 6th thoracic vertebra compressing the spinal cord, especially around weeks 5 (broilers) to 13 (broiler breeders), and increased mortality (Stalker et al. 2010; Makrai et al. 2011; Borst et al. 2012). It is assumed that EC persists in the environment (De Herdt et al. 2009) and may infect subsequent broiler production cycles. Nonetheless, the source in the broiler houses has not been found yet (Robbins et al. 2012; Borst et al. 2017). Consecutive outbreaks of pathogenic EC imply a farm-associated environmental or biologic reservoir (De Herdt et al. 2009).

Tenacity can be defined as the robustness of microorganisms to defined exogenous factors (Von Sprockhoff 1979). In the Anglo-American language, the term ‘tenacity’ is uncommon; instead, terms such as ‘resistance’, ‘sensitivity’ or ‘survival’ are used (Egen 2000). Survival can be understood as keeping the viability under disadvantageous circumstances (Roszak and Colwell 1987).
Enterococcus sp. generally have the ability to tolerate low temperatures as 5 or 15°C or rather these favour their survival (Cools et al. 2001). However, many enterococci can also endure high temperatures as 45 up to 60°C, which distinguishes them from other closely related genera such as Streptococci (Moreno et al. 2006). For example, Enterococcus faecalis and Enterococcus faecium were frequently isolated from environmental samples collected in poultry houses and from samples of affected birds such as tissues and swabs (Dolka et al. 2017). They persisted in the environment, even under unfavourable conditions such as high temperature, drought or chemicals (Bradley and Fraise 1996; Cools et al. 2001; Liu et al. 2018). Until now, not much is known about the resistance of EC to environmental factors. The only source, which examined the survival of EC until now, demonstrated that it can grow at low temperatures (4, 10°C) and survives at 60°C for 1 h and some strains at 70°C for 15–30 min (Dolka et al. 2016).

Air humidity is known to affect the survival of bacteria in the environment (Gundermann 1972). Observations linking air humidity and the survival of EC are not available. Other Enterococcus species show resistance to environmental stresses, such as demonstrated for E. faecalis to desiccation with survival times of more than 60 days (Hartel et al. 2005; Lebreton et al. 2017). There are contradictory findings for Enterococcus sp. On the one hand, these bacteria can persist for more than 11 weeks under desiccation (Bale et al. 1993). On the other hand, another study claims that drought stress leads to population decline in Enterococcus sp. (Cools et al. 2001).

Different substrates are found in poultry houses. Polyvinyl chloride (PVC) is a polymer and one of the most used synthetic plastics in the world (Vesterberg et al. 2005). It can be found in drinker cups and feeders in poultry houses. Survival of E. faecalis and E. faecium isolated from humans and the environment of hospitals was observed on PVC for up to 4 months (Wendt et al. 1998). Broiler litter was investigated for its microbial composition by culture and molecular detection methods (Lu et al. 2003). On evaluating the total aerobic bacterial counts, enteric bacteria like enterococci accounted for only 0.1%. When analysing 16S rDNA sequences, 2% of the sequences were assigned to the group of Enterococcaceae, including EC. Litter can be described as a mix of feather particles, faeces, feed and bedding components such as wood shavings in broiler houses (Kuntz et al. 2004). Enterococcus faecalis was rarely isolated from broiler litter with fewer than 2% of the isolates, but a high number of unspecified enterococci were detected. Enterococci such as E. faecalis, E. faecium and other unspecified Enterococcus sp. were isolated from poultry litter over a 120-day period, indicating high persistence in that matrix (Graham et al. 2009).

Dust is very common in poultry houses and originates from feed, feather components, bedding material and faeces (Carpenter 1986). Decay, amount, composition and formation of airborne particles like dust are influenced by many factors such as temperature and air humidity. Most bacterial micro-organisms isolated from dust are Gram-positive cocci (Hartung and Saleh 2007). Until now, EC was not isolated from dust yet. However, it is discussed to invade via the respiratory tract (Kense and Landman 2011; Jung and Rautenschlein 2014) so that an infection via inhaled dust particles could be theoretically possible (Kense and Landman 2012; Jung et al. 2018).

The aim of the present study was to characterize the survival of specific EC isolates on litter and PVC at different air humidities (32% RH, 78% RH) and temperatures (15, 25, 37°C), which can be found in the environment of broiler chickens at different stages of the production cycle.

Materials and methods

Bacterial strains

For the tenacity studies, we used the pathogenic EC isolate 14/086/4/A (Jung et al. 2017a), designated as EC14 in the following. The strain was isolated from the heart of a broiler with EC septicaemia. For selected substrate–temperature–air humidity combinations, the commensal EC strain 13/655/3/B (EC13), which was isolated from the intestine of a broiler chicken from a production cycle without classical EC infection, was also investigated (Jung et al. 2017a). In addition, two other pathogenic strains, 15/827/1/A (EC15) and 14/166/2/A (EC14.2) (Jung et al. 2017a), both isolated from the heart of the chickens, were tested in one selected combination of environmental parameters (PVC, 25°C, 32% RH). All strains were stored at −80°C using the cryobank system (Mast Diagnostica GmbH, Reinfeld, Germany). Before each experiment, one bead was thawed, plated on Columbia Agar with Sheep Blood (COLSB; Oxoid Deutschland GmbH, Wesel, Germany) and incubated in a CO₂-enriched atmosphere at 37°C for 24 h. Bacterial colonies of this plate were further used for subcultivation on a COLSB plate followed by another incubation step. Subsequently, bacterial colonies were suspended in sterile physiologic saline solution in a flat-bottom glass test tube with a SteriStopper® (Heinz Herzen Medizinalbedarf GmbH, Hamburg, Germany), diluted to a McFarland standard of 3.3 (10⁸ CFU per ml) and used for spiking the substrates.
Substrates

PVC (white PVC, Alt Industriebedarf, Neresheim, Germany) was cut into pieces of 1.0 × 1.0 × 0.1 cm, washed three times with distilled water, rinsed with 70% ethanol and dried in a laminar flow cabinet until inoculation (modified according to Egen 2000).

Litter–faeces mixture (‘litter’; bedding consisting of wood shavings) and dust were collected in a broiler house (Ross 308) during different production cycles on the same farm in Lower Saxony, Germany at the third or fourth week of life. The litter was always collected at five locations of a fictive line near the walls on both sides and of another fictive line in the centre of the broiler house. Dust was obtained from window ledges, heating installations and pipelines at different locations.

The litter was pooled and shredded by a commercial kitchen blender (model number 17956-56; Russell Hobbs Essentials Ltd, Manchester, UK), filled in plastic bags and autoclaved at 121°C for 15 min. The pooled dust was filtered with a sieve (1.0 × 1.0 mm opening), filled in 500 ml bottles and also autoclaved just like the litter at 121°C for 15 min. Aliquots of 1.0 g litter or 0.5 g dust were weighed in glass test tubes with Steristoppers® and again autoclaved before use.

Inoculation

In a laminar flow cabinet, PVC plates were placed on blotting paper. The PVC samples were inoculated each with 10 µl (10⁹ CFU) of the bacterial suspension. After 1 h of drying, PVC plates were transferred to glass test tubes and the tubes were sealed with Steristoppers (modified according to Egen 2000). For litter and dust, aliquots of 1.0 or 0.5 g were inoculated with 10 µl (10⁹ CFU) of the bacterial suspension, dried in the open glass test tubes for 1 h and then sealed likewise. For each sampling time point, test tubes were processed in triplicate. Each trial included one sample inoculated with 10 µl of sterile physiologic saline solution and another non-inoculated sample of the respective substrate as negative controls.

Experimental setup

All glass test tubes were transferred to desiccators (DWK Life Sciences GmbH, Wertheim, Germany) with a defined microclimate. Two different air humidities were created by saturated saline solutions (O’Brien 1948; OIML 1996; Gillespie et al. 2000; Lu and Chen 2007). To establish an air humidity of 32% RH, a saturated solution of anhydrous calcium chloride (AppliChem GmbH, Darmstadt, Germany) in water was prepared (O’Brien 1948), autoclaved at 121°C for 15 min and filled in a desiccator. The filled desiccator was pre-incubated for at least 72 h before the placement of samples (Gundermann 1972; Cimiotti 1980). The relative air humidity was confirmed using a digital hygrometer (TFA Dostmann GmbH & Co. KG, Wertheim-Reicholzheim, Germany). Sodium chloride (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was used likewise to generate an air humidity of 78% RH. For every substrate and air humidity, three different temperatures, 15, 25 and 37°C, were tested (Cimiotti 1980; Egen 2000). The applied conditions were inspired by possible environmental conditions in a broiler production cycle (Aviagen 2018, accessed July 2020). Although the typical parameter during a production cycle range between 50 and 70% RH for the relative air humidity and 29–31 to 20°C for the temperature (Aviagen 2018, accessed July 2020), we tried to cover the whole range of environmental conditions to illustrate the potential differences more clearly.

An overview about all used temperature–humidity–substrate–strain combinations is given in Table 1.

Samples were removed in triplicate from the desiccator at defined time points including at least 0, 24, 48, 72, 96 and 120 h post-inoculation for each trial. A set of three samples was processed directly after inoculation and 1 h drying time before the placement in a desiccator as the time point ‘0 h’. Additional time points (up to 4272 h) were included based on previous trials, but chosen individually per sample and course of the trial (see also Table S1).

Determination of total viable count

Each glass test tube was filled with 3 ml (litter, dust) or 1 ml (PVC) of sterile physiologic saline solution (Cimiotti 1980; Egen 2000). The tubes were shaken at a G-force of 2 g at room temperature for 15 min. After shaking, a 10-fold serial dilution for each sample was prepared. 100 µl of each dilution was plated on COLSB plates in duplicate and incubated at 37°C for 48 h in a CO₂-enriched atmosphere. One test tube of each negative control was processed in a similar manner at the first sampling date. After incubation, colony forming units (CFU) of all evaluable dilution levels were counted with ProtoCOL3 (Synbiosis, Cambridge, UK) and for each dilution level, count per millilitre was calculated. The count limit was set at 10–300 CFU per counting frame. The mean value for each time point was calculated from all counts (n = 3) of the sample dilution level.

Data analysis

For statistical analysis, mean values per time point were calculated, logarithmized and plotted against time with GraphPad prism 8 (GraphPad Software Inc., San Diego, CA). Using linear regression models developed by
The measured bacterial surviving time ranged from 48 to 4272 h (178 days) for EC14 depending on the substrate–humidity–temperature combination (Table 1). Using the linear regression model, even longer survival times up to over 19 000 h (on litter) could be calculated in consideration of the coefficient of determination.

### Litter

The shortest survival time of EC14 on litter was detected at 37°C and 32% RH with 120 h, followed by the conditions of 37°C and 78% RH with a survival time of 168 h (Fig. 1a, Table 1). Comparing the temperatures, EC14 survived generally for the shortest time at 37°C on litter. At 25°C, EC14 could be isolated for a longer time with a measured survival time of up to over 3000 h, depending on the relative air humidity (Fig. 1b). In contrast to 37°C, EC14 survived longer at a relative air humidity of 32% RH instead of 78% RH at 25°C with a time difference of over 500 h between the last measured time points (Table 1). The longest survival on litter was at 15°C (Fig. 1c). Survival times of 2784 h (78% RH) and 4272 h (32% RH) were measured before the trials had to be finished (Table 1). Similar to 25°C, EC14 was detected for a longer time at 32% RH than at 78% RH with a time difference of over 1400 h (Fig. 1c).

### Results

The measured bacterial surviving time ranged from 48 to 4272 h (178 days) for EC14 depending on the substrate–humidity–temperature combination (Table 1). Using the linear regression model, even longer survival times up to over 19 000 h (on litter) could be calculated in consideration of the coefficient of determination.
Concerning the second tested substrate PVC, the shortest survival time of EC14 of all tested substrates was measured with 48 h at 37°C and 32% RH (Fig. 2a, Table 1). At 78% RH and at the same temperature, EC14 could be detected 24 h longer (Fig. 2a). At 25°C, the bacteria could be isolated from the samples up until 240 h (78% RH) and 336 h (32% RH), respectively (Fig. 2b). The longest survival time was found at 15°C with 432 h (78% RH) and 2280 h (32% RH), respectively (Fig. 2c, Table 1). At 15°C and 78% RH, a higher number of CFU per ml in comparison to the other trials on PVC (3 Log_{10} CFU per ml compared to about 2 Log_{10} CFU per ml) was isolated at the last measurable time point (Fig. 2c). As for litter, the survival time of EC14 on PVC was only longer at the higher relative air humidity (78% RH) when in combination with the highest tested temperature of 37°C (Fig. 2a–c). At 25 and 15°C, EC14 showed longer survival at the lower relative air humidity with 32% RH compared to the higher one of 78% RH. A time difference of 96 h at 25°C and 1848 h at 15°C was measured between the last measured time points of the different relative air humidities (Table 1). Overall, the enterococcal survival time on PVC showed a difference of over 2200 h between 15°C with 32% RH (2280 h) and 37°C with 32% RH (48 h; Table 1).

**Figure 1** Enterococcus cecorum isolate 14/086/4/A (EC14, pathogenic) survival (log_{10} CFU per ml; mean of triplicate samples) plotted over time. Grouped by the factor ‘litter’ when exposed to temperatures at different relative air humidities. Trials marked with * were finished before complete EC die-off. (a) 37°C, (b) 25°C, (c) 15°C. Relative air humidity: ■ 78% RH, □ 32% RH.
For dust, two trials were conducted with the strain EC14 (Fig. 3). EC14 survived for 120 h at 37°C and 78% RH (Fig. 3). At 15°C and 32% RH, the trial was finished at 2784 h (Fig. 3). The time differences between the last measured time points were more than 2664 h (Table 1).

Comparisons of substrates
Overall, the longest survival time of EC14 was detected on litter, followed by dust and then PVC depending on the combination of temperature and relative air humidity (Table 1). The significant differences per time point between the substrates are summarized in Table S1 ($P \leq 0.05$).

Comparisons of EC strains
Different EC strains were tested at specific conditions (Figs. 4 and 5). The pathogenic EC isolate 14/086/4/A (EC14) was compared with the non-pathogenic EC isolate 13/655/3/B (EC13) on PVC and litter at 25°C and two different relative air humidities (Fig. 4). The pathogenic EC14 survived longer regardless of the conditions (Fig. 4). At 25°C and 32% RH, EC14 was isolated up to

![Graph](https://via.placeholder.com/150)

Figure 2. Enterococcus cecorum isolate 14/086/4/A (EC14, pathogenic) survival (log$_{10}$ CFU per ml; mean of triplicate samples) plotted over time. Grouped by the factor 'PVC' when exposed to temperatures at different relative air humidities. Trials marked with * were finished before complete EC die-off. (a) 37°C, (b) 25°C, (c) 15°C. Relative air humidity: ■ 78% RH, □ 32% RH.
3288 h on litter, whereas the non-pathogenic EC13 was detected up to 2784 h at the same conditions (Fig. 4a). At the same relative air humidity and temperature on PVC, EC14 was detected 240 h longer than EC13 (Fig. 4b, Table 1). At 78% RH and on litter, EC14 survived for 600 h and EC13 for 96 h (Fig. 4c), the last measured time points differing over 500 h. At 25°C and 32% RH, both strains survived longer on litter than on PVC with a difference of 2952 h (EC14) and 2688 h (EC13) between the last measured time points (Fig. 4a,b, Table 1). At 25°C and on litter, both isolates were detected longer at 32% RH than at 78% RH, with a time difference of 2688 h for each strain (Fig. 4a,c).

At 25°C and 32% RH, both strains (EC14 and EC13) and another two pathogenic EC strains EC14-2 and EC15 were tested on PVC (Fig. 5). The measured survival time for EC15 as well as for EC14 amounted to 432 h, but more CFU per ml of EC15 were detected at the last measured time point (Fig. 5). The strain EC14-2 survived for 336 h, followed by the non-pathogenic EC13 with 168 h (Fig. 5).

Again, significant differences per time point between the substrates are summarized in Table S1 (P ≤ 0.05). When comparing the pathogenic strain EC14 and the non-pathogenic strain EC13, the significant differences per time point did not display a consistent pattern (P ≤ 0.05). At all time points, pathogenic EC15 was significantly different from the two other pathogenic and one non-pathogenic strains (P ≤ 0.05).

Discussion

We investigated the ability of different EC strains to survive at different temperatures and relative air humidities on PVC, litter and dust. These substrates are commonly found in broiler flocks. We tested the enterococcal survival time not only for one pathogenic strain but also for one non-pathogenic and two other pathogenic strains at selected conditions. To our knowledge, this is the first extensive study focusing on the survival of EC under different environmental conditions.

Temperature and relative air humidity

Regarding temperature, it was generally found that the lower the temperature, the longer the survival time of EC14. This is in accordance with the literature, where it is postulated for enterococci to survive better at low than at high temperatures (Cools et al. 2001; Dolka et al. 2016). The temperature was a very important influencing factor and significantly affected the amount of surviving bacteria at nearly all of the measurable and calculable time points (Table S1). These findings suggest that the bacterial survival time is to a large extent dependent on the environmental temperature.

In comparison to other bacteria, enterococci such as EC have high durability. One study compared the survival of E. coli with the survival of Enterococcus sp. (Cools et al. 2001). Escherichia coli could survive for up to 68 days at 5°C (Cools et al. 2001). In contrast, Enterococcus sp. remained constant at 5°C for a period of 80 days (Cools et al. 2001) and in our study EC could be detected for 18 days (432 h) up to 178 days (4272 h) at 15°C depending on the substrate and the relative air humidity. Bacteria as E. coli showed also a longer survival time at lower temperatures as demonstrated in our study for EC14 (Milling et al. 2005; Moretro et al. 2010; Chen et al. 2018). For example, E. coli could be isolated for only 7 h on pine-wood sawdust at 37°C, but for over 144 h (6 days) at 4°C (Milling et al. 2005). On plastic chips, survival times of over 144 h for every temperature were shown, but the bacterial titre was over 3 Log10 CFU per gram higher for 4°C compared to 37°C at the last measured time point (Milling et al. 2005). The same applies to Salmonella sp. (Pietronave et al. 2004; Vinnerås 2007; Chen et al. 2018). In composts, it can be found for over 168 days at 5°C and for up to 91 days at 22°C depending on the inoculum level (Chen et al. 2018). In manure, no significant reduction of Salmonella sp. was found at 4°C, but the decimal reduction of 25 days was shown at 14°C (Vinnerås 2007). However, it has to be considered that the bacterial sensitivity to low temperatures varies widely and is based on bacterial population...
density, growth temperature, cooling rate and the temperature range at which cooling occurs (Postgate and Hunter 1963; Mackey 1984; Wesche et al. 2009).

For the relative air humidity, the tendency was partly dependent on the temperature. EC14 was inactivated faster at the high relative air humidity (78% RH) in combination with the temperatures 15 and 25°C. Only at 37°C was the EC decrease faster at the low relative air humidity in contrast to the other results. Enterococci are known to be able to survive under dry conditions (Bale et al. 1993; Hartel et al. 2005; Lebreton et al. 2017), but drought stress should also lead to a population decline in enterococci (Cools et al. 2001). The results of our study are in accordance with these former findings when taking into consideration the different survival times under various environmental conditions. However, since especially non-pathogenic EC strains are known as gut commensals in chickens, an explanation could be that EC can survive under conditions similar to the intestinal environment with a high relative humidity (RH) such as 78% RH and at temperatures such as 37°C, which is in proximity to the chicken body temperature of 40°C (Lidwell and Lowbury 1950; Menezes-Blackburn et al. 2015).

Similar to enterococci, total inactivation of *E. faecalis* was observed at a RH close to 85% RH and 25°C in 30 min (Robine et al. 2000). The same trend was found for *Bordetella avium* and *Campylobacter jejuni* (Cimiotti 1980; Egen 2000). In contrast, other bacteria such as *Salmonella* sp. survived longer under humid conditions (Stine et al. 2005; Blessington et al. 2014; Margas et al. 2014). For *E. coli*, there is contradictory information regarding its survival time under different relative humidities (Milling et al. 2005; Stine et al. 2005; Moretro et al. 2010).

**Figure 4** *Enterococcus cecorum* survival (log_{10} CFU per ml; mean of triplicate samples) plotted over time. Grouped by the factor ‘strain’ when exposed to 25°C. Trials marked with * were finished before complete EC die-off. (a) Litter and 32% RH, (b) PVC and 32% RH, (c) Litter and 78% RH. *Enterococcus cecorum* (EC) strains: ■ EC isolate 14/086/4/A (EC14, pathogenic), ○ EC isolate 13/655/3/B (EC13, non-pathogenic).
Exposed to 25°C and 32% RH on PVC. EC strains: ○ EC isolate 15/827/1/A (EC15, pathogenic), □ EC isolate 14/086/4/A (EC14, pathogenic), ● EC isolate 14/166/2/A (EC14.2, pathogenic), ○ EC isolate 13/655/3/B (EC13, non-pathogenic).

Substrates

Besides the tested substrates (litter, PVC, dust) in our study, there are also other substrates such as concrete floor, stainless steel on the drinking and feeding lines along with PVC, chick paper at the first days, bricks on the walls and optionally wood or plastics other than PVC in broiler houses. We only selected the most common substrates found in nearly all broiler houses. For the other substrates, our data could indicate a positive or negative impact on the survival of EC because of some similar characteristics of several substrates. However, additional trials have to be conducted to obtain detailed and substantiated data on the ability of EC to survive on those substrates. It is known for other enterococci that they could be isolated from walls and floors of broiler houses (Borgen et al. 2000). Isolation of E. faecium from other substrates such as feed equipment, the floor and walls, heaters, wooden partitions and scales in broiler houses was also successful (Garcia-Migura et al. 2007). Vancomycin-resistant E. faecalis and E. faecium survived up to several weeks on stainless steel, whereas on copper alloys, only a short survival time of 1 h was detected (Robine et al. 2000; Warnes et al. 2010).

When comparing the survival time of the tested EC strains on our tested substrates, this was the longest on litter and the shortest on PVC. Dust as the third tested substrate in our trials was excluded for several temperature–humidity combinations because the measured survival times of EC14 on dust were located between those of PVC and litter at the same conditions. One possible reason for the different survival times may be the different texture of these substrates (Heijnen et al. 1992; England et al. 1993). On a smooth surface like PVC, the bacteria are more exposed to the environmental conditions than on a substrate like litter consisting of many small particles. The risk of air-drying damage on substrates like PVC could be higher, for example (Potts 1994; Billi and Potts 2002). Another possible reason may be the different water absorption ability of the substrates (Billi and Potts 2002; Hanczvikkel and Tóth 2018). PVC as a synthetic plastic has a very low ability to absorb and even store water (Kiani et al. 2011; Rimdusit et al. 2012). In contrast, litter can absorb it easily and deposit it (Deininger et al. 2000; Miles et al. 2011; Dunlop et al. 2015). Consequently, bacteria on litter most likely have a higher amount of essential water available than on PVC and thus can survive longer. The nutrient amount is another point for the survival of the enterococci, especially on used litter (Wehunt et al. 1960; Patterson et al. 1998). Other authors confirmed a longer persistence of bacteria in the presence of organic components (Mallmann and Litsky 1951; Hirai 1991; Jawad et al. 1996). Additionally, it was suggested that a low survival rate of E. faecalis on PVC could be due to the high reactivity of PVC, with the presence of oxidizing sites, the release of hydrochloric acid and the presence of additives (Robine et al. 2000). Another point could be the influence of the dissemination of the inoculum on the survival time including different surface tensions on the various substrates and the absorption of the inoculum (Egen 2000).

Due to the potential resistance of the tested EC strains, trials lasting unexpectedly longer than 2000 h had to be terminated in our experiments. However, the linear regression model was used to assume the final die-off of the tested EC strains (Table 1). The calculated survival times of the tested EC strains ranged from over 71 h to nearly 20 000 h (over 2 years). The greatest difference between the calculated and the measured survival time amounted to nearly 15 000 h under the conditions with the longest survival time at 15°C and 32% RH on litter. To explain this vast difference, the associated coefficients of determination were calculated. These varied between 0·483 and 0·988, indicating a variable goodness of fit. For all but one of the terminated trials, the coefficient of determination reached over 0·7 or even over 0·9 so that the linear regression model had an acceptable goodness of fit. Nevertheless, linear regression is not always the most suitable model to calculate bacterial survival as already discussed in previous sources (Geeraerd et al. 2005).

Figure 5 Survival of different Enterococcus cecorum (EC) strains (log₁₀ CFU per ml; mean of triplicate samples) plotted over time. Exposed to 25°C and 32% RH on PVC. EC strains: ○ EC isolate 15/827/1/A (EC15, pathogenic), □ EC isolate 14/086/4/A (EC14, pathogenic), ● EC isolate 14/166/2/A (EC14.2, pathogenic), ○ EC isolate 13/655/3/B (EC13, non-pathogenic).
After inoculation of the substrates, an initial 1-h drying procedure of the inoculum was conducted. This initial drying procedure reduces the amount of CFU in the sample, which was also reported by other researchers (Wendt et al. 1998; Egen 2000; Redfern and Verran 2017). For compensation of the different drying losses and comparison of the different trials, the CFU per ml recovered from the substrate samples directly after the drying time were logarithmized and percentage change values of these data were used for further analysis (Table S1).

Furthermore, it has to be considered that for determining viable counts, a culture-dependent method was used. Some enterococci like E. faecalis, E. hirae and E. faecium are known to be able to enter a viable but non-culturable (VBNC) state in response to environmental stress (Lleó et al. 2001; Oliver 2010; Gin and Goh 2013). However, this has not been investigated for EC yet. Consequently, it has to be taken into consideration that EC could have entered this state at some point during the trials and was no longer detectable by the used methods anymore. Other methods like live staining may be of interest for further research (Heim et al. 2002).

EC strains

On comparing the different EC strains at the same conditions, the three pathogenic EC strains survived longer than the non-pathogenic one. Differences between the pathogenic strains were found regarding longer survival time or higher isolation rate. Therefore, we conclude that the pathogenicity may have an influence on the survival ability of EC.

Survival and/or multiplication of different bacteria in protected sites was correlated with pathogenicity (Wilson et al. 1999). A correlation between virulence and durability for bacterial and viral pathogens such as Bordetella pertussis, Streptococcus pneumoniae or influenza virus is occasionally reported (Dennis and Lee 1988; Walther and Ewald 2004). A possible explanation could be the genetic diversity among the pathogenic strains (Potts 1994; Gastmeier et al. 2006). In contrast, other authors do not assume a correlation as they found no differences (Wendt et al. 1998; Neely and Maley 2000; Kramer et al. 2006). For enterococci, a detailed study about such a potential correlation was not conducted yet. The mentioned genetic diversity was also found for E. faecalis (McBrine et al. 2007), but not necessarily for EC (Jung et al. 2018). There are some reports of diversity among pathogenic EC, but a greater similarity among the pathogenic strains in comparison to the commensal strains is assumed (Wijetunge et al. 2012; Jung et al. 2018). However, two enterococcal traits are known, that is, a relatively high durability (Bale et al. 1993; Cools et al. 2001; Hartel et al. 2005; Lebreton et al. 2017) and the frequent genetic exchange of putative virulence factors via conjugative plasmids and transposons (Descheemaeker et al. 1999; Angulo et al. 2006; Hammerum 2012; Jung et al. 2018). Such genes typically confer traits that provide survival advantages to organisms in unusual environments such as virulence factors (Eberhard 1989; Jett et al. 1994). The ability to exchange genes itself can be considered an expression of virulence (Jett et al. 1994). Consequently, a correlation of the high durability and the pathogenicity or virulence of enterococci could be possible.

In broiler houses, a relative air humidity of even under 25% RH can be found in houses with wholehouse heating and nipple drinkers, although a higher relative air humidity of about 60% RH ± 10% RH depending on the temperature are recommended (Aviagen 2018, accessed July 2020). As mentioned before, the typical parameter during a production cycle range between 50 and 70% RH for the relative air humidity and 29–31°C to 20°C for the temperature considering the age of the broilers (Aviagen 2018, accessed July 2020). The older the broilers, the colder and drier the environmental conditions should be. In this study, also conditions beyond the recommended profile were tested to illustrate the potential influences of the parameters on the survival of EC more clearly. Additionally, in this context not only parameters during the housing of the broilers but also in the downtime between the cycles may be important. The demonstrated possible longevity of pathogenic EC strains can be a risk for carry-over and infection of subsequent broiler cycles in the same house, particularly when cleaning and disinfection are performed inadequately (Heuer et al. 2002). Therefore, the choice of cleaning and disinfection agents and procedure is important. Since a high temperature with a low relative air humidity over a couple of days is disadvantageous for EC, these conditions may be useful to consider when aiming to eliminate EC in broiler houses. At the beginning of a broiler production cycle, it is recommended to preheat the broiler house to achieve an air temperature of around 30°C with a RH of 60–70% RH (Aviagen 2018, accessed July 2020). These environmental conditions are partly similar to those in this study. At conditions of 25 or 37°C with 78% RH, EC survived between 72 and 600 h, depending on the substrate. Hence, if a carry-over of EC occurs, the bacteria will survive long enough in the preheated house to infect the broiler chickens. By prolonging the vacancy period between two broiler production cycles in combination with disadvantageous conditions, a better control of EC outbreaks may be possible. Further research is needed to test these considerations.
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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Significant $P \leq 0.05$ differences at sampling time points (h) for the tested combinations of substrate, temperature and relative air humidity for a non pathogenic (EC13) and pathogenic (EC14, EC14.2, EC15) Enterococcus cecorum strains with regard to the presented figures.