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Determination of the microbiological load of poultry drinking water before and after sanitation by culture based and sequence-based methods

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ABBREVIATIONS

%	**Percent**
°C	Degree Celsius
§	Paragraph
µl	Microliter
µM	Micromole
LMSVG	Lebensmittelsicherheits- und Verbraucherschutzgesetz
AMC	Aerobic cell count
aq	aqueous
AS	After sanitation
BGBl.	Bundesgesetzblatt
BHI	Brain Heart Broth
BPW	Buffered peptone water
BS	Before sanitation
C.	*Campylobacter*
CCDA	Charcoal-Cefoperozone-Deoxycholate-Agar
CFU	Colony forming units
Cl_2	Chlorine
ClO_2	Chlorine dioxide
DNA	Deoxyribonucleic acid
DEPC	Diethylpyrocarbonate
dNTPs	Desoxynucleotide triphosphates
e-	Electron
E.	*Escherichia*
EB	*Enterobacteriaceae*
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EU	European Union
FFoQSI	Feed and Food Quality Safety and Innovation
g	Gram
GIT	Gastrointestinal tract
h	Hour(s)
HAT	Hyper-aerotolerant
HCl	Hydrochloric acid
ISO	International Organization for Standardization
l	Liter
log	Logarithm
M	Mole
mg	Milligram
MKTTn	Muller-Kaufmann Tetrathionate Novobiocin
ml	Milliliter
mM	Millimole
nM	Nanomole
Nr.	Number
PCR	Polymerase-Chain Reaction

pH	pH - value
ppm	Parts per million
PS	*Pseudomonadaceae*
rcf	Relative centrifugal force
rpm	Rotations per minute
rRNA	Ribosomal Ribonucleic acid
RVS	Rappaport-Vassiliadis soy peptone broth
S.	*Salmonella*
Spp.	Several species
TAE	Trisaminomethane acetate-ethylenediaminetetraacetic acid
Tris	Trishydroxymethylaminomethan
TSAY	Trypto-Casein-Soy-Agar
U	Unit
VD	Dilution
VRBD	Violet-Red-Bile-Dextrose-Agar
XLD	Xylose-Lysine-Desoxycholate Agar

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1. INTRODUCTION

1.1. HYGIENE IN POULTRY FARMS

Caring for a high quality in poultry drinking water is of high importance since chickens consume twice as much water as feed (https://www.thepoultrysite.com/articles/waterrelated-factors-in-broiler-production; accessed on: 07-03-2022).

More than often, broiler farms observe weak flock performance and sanitary issues affecting the overall health, which can be often linked to poor water management (https://www.biotecharticles.com /Agriculture-Article/Water-Quality-for-Poultry-Birds-3630.html; accessed on: 07-03-2022). Water drinking systems like nipple drinkers initially improved the overall water hygiene but workers became more reluctant to monitoring the quality since those water systems are closed and it became harder to examine them visually (MAHARJAN et al., 2016). *Escherichia coli* and *Pseudomonadaceae* were commonly found in water systems, whereas *E. coli* contaminated water speaks for fecal contamination (https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/coliform-bacteria; accessed on: 07-03-2022; HALKMAN et al., 2014; LI et al., 2019).

S*almonella* and *Campylobacter* were often detected in water tanks and drinking water systems (COOLS et al., 2003). Many surface waters directly impact the groundwater quality. *Salmonella* for instance have shown to be quite resistant in aquatic environments (LIU et al., 2018). Thus, regular water sanitation practices have a big impact on animal health such as prevention of biofilm development, which would expose broiler chickens to more pathogens (MAHARJAN et al., 2016).

Moderate to severe leg lesions in poultry farms have been described in the Netherlands due to exposure of wet litter resulting in footpad dermatitis and hock burns (DE JONG et al., 2014). Drinking water contaminated with microorganisms could lead to a chronic stress response due to releasing stress related hormones like corticosterone through the adrenal glands (ŠKRBIĆ et al., 2015). Hyperplasia and hypertrophy of adrenal glands may appear in broiler chickens whereas the left adrenal gland seems to react more sensitive which would mean that a slight asymmetry of both glands compared could be experienced (JACOBS et al., 2020).

1.2. DRINKING WATER SANITATION

Disinfectants like sodium hypochlorite, chlorine gas and calcium hypochlorite are often utilized in poultry farms in order to sanitize drinking water. Low pH levels benefit the sanitary effect of chlorination (https://www.wateronline.com/doc/disinfection-of-poultry-drinking-water-and-pr-0001; http://www.positiveaction.info/pdfs/articles/hp31_3p21.pdf; accessed on: 07-03-2022). Sometimes drinking water might even be acidified to enhance the sanitary effect, but it is important that

chlorine and acidifiers should be blended separately in order to circumvent the development of toxic gas. Observations have shown that 2-5 parts per million (ppm) of free chlorine remains have an effect against the majority of microorganisms whereas levels above 200 ppm showed to have a poisonous effect. Using chlorine in drinking water showed to be effective against *E. coli*, Influenza-A-Virus H5N1, *Campylobacter* and enterococci (MOHAMMED et al., 2020).

Chlorine dioxide seems to be a better option for drinking water sanitation since it does not change the flavor or scent. The chemical reaction of the electron transferring mechanism is quite simple since a single electron is transferred and reduced to chlorite ion: $ClO_2(aq) + e- => ClO_2-$

In addition, chlorine dioxide eliminates even bacteria and viruses better than chlorine's counterparts (like hydrogen peroxide, quaternary ammonium compounds and iodophores) and is unaffected by a wide range of pH differences (MICCICHE et al., 2018). Biofilms can be linked to various issues when it comes to water pipeline sanitation. Chlorine based and peroxide based disinfectants are not as effective against microorganisms because they are unable to penetrate the biofilm matrix (MAHARJAN et al., 2016). Using disinfectants like chlorine dioxide (ClO_2) have shown to be more effective in order to remove biofilms (JACOBS et al., 2020). Unpleasantly, microorganisms such as *Salmonella* or *Campylobacter* seem to adapt and become more resistant to ClO_2 because the application concentration was too low (MAHARJAN et al., 2016).

Furthermore, acidified drinking water has shown to significantly decrease the indirect transmission of *Campylobacter* (BUNNIK et al., 2018). Effects showed a reduction in the total aerobic cell count (AMC) of the cecum as well as a general improvement in weight gain (HAMID et al., 2018).

1.3. PATHOGENS IN DRINKING WATER

Contaminated drinking water can be an important source in a high-speed spread of bacterial or viral diseases (https://www.thepoultrysite.com/articles/water-quality-and-broiler-performance; https://www.oie.int/fileadmin/Home/eng/Health_standards/tahc/current/chapitre_prevent_salmonella.pdf; accessed on: 07-03-2022). *Salmonella, E. coli* and *Campylobacter* spp. have the highest relevance in poultry farms (https://www.safepoultry.com/controlprogram_onthefarms.aspx; accessed on: 07-03-2022). These pathogens are most commonly found in the gastrointestinal tract, source for different illnesses and might elevate rates of morbidity and mortality (HAKEEM et al., 2020). *E. coli* is considered as a fecal indicator as a representative commensal of the poultry intestinal tract. *E. coli* is responsible for several diseases such as „yolk-sac infection, enteritis air-sac disease, omphalitis or coligranuloma perihepatitis, colibacillosis“(CHRISTENSEN et al., 2021; ZAMAN et al., 2012).

The most frequently isolated *Salmonella* serovars in poultry are *Salmonella typhimurium* and *Salmonella enteritidis*, which may cause foodborne diseases in humans (ANDOH et al., 2016).

Salmonella infantis (42.46%), *Salmonella mbandaka* (9.94%) and *S. typhimurium* (9.43%) are most commonly found in broiler poultry farms (https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2019.5596; accessed on: 07-03-2022).

C. coli and C. jejuni, the most commonly detected species in intestinal convolutes and neck skin samples in slaughterhouses, are often associated with stress and animal welfare issues (IANNETTI et al., 2020). EFSA and ECDC have provided information, that *Campylobacter* with an EU notification rate of 59.7 per 100,000 population was responsible for the majority of gastrointestinal diseases in 2019. Of these *Campylobacter* infections, "83.1% were *C. jejuni*, 10.8% *C. coli*, 0.1% *C. lari*, 0.1% *C. fetus* and 0.1% *C. upsaliensis* (https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2021.6406; accessed on: 07-03-2022*)."*

Campylobacter is able to penetrate deep into the cracks as they provide the perfect environment for bacteria to settle, multiply and form biofilms as they are protected from cold (ROSSI et al., 2017). Both *Salmonella* and *Campylobacter* are responsible for a high amount of gastrointestinal diseases, mainly diarrhea worldwide (https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2020.6090; accessed on: 07-03-2022). *Campylobacter* and *Salmonella* can persist in young children, which could have a long-term negative impact on their nutritional health. Research has shown that hyper-aerotolerant (HAT) *C. jejuni* are mainly prominent in retail poultry meat. Compared to aero-sensitive *C. jejuni* strains, HAT *C. jejuni* would survive a significantly longer time at 4°C in raw poultry meat under aerobic conditions (OH et al., 2017). Therefore, removing the intestines during slaughtering has been linked to the highest risk of contamination. Cross-contamination also seems to have an impact on spreading this disease (KAGAMBEGA et al., 2018).

1.4. LEGAL BASIS

The water law 1959 – WRG 1959, BGBl. Nr. 215/1959 regulates the usage and protection of water. The basic hygiene requirements for the production and treatment of food are contained in the EU regulation (EG) No. 852/2004 and the regulation (EG) No. 853/2004 containing specific hygiene regulations for food of animal origin. The Lebensmittelsicherheits- und Verbraucherschutzgesetz – LMSVG, BGBl. I Nr. 13/2006 regulates placing water for human use (drinking water) on the market. According to §3 Abs. 2 drinking water for human use can be water from a drinking fountain to the consumer for usage as groceries or in a food company. Further requirements like placing products on the market, quality and inspection for human use is regulated in (Trinkwasserverordnung – TWV), BGBl. II Nr. 304/2001. Chapter B1 „Trinkwasser" of the Codex Alimentarius Austriacus explains quality criterias for drinking water and contains additions which go beyond the regulation. The Codex

Alimentarius Austriacus finds its legal basis in the §76 of the „Lebensmittelsicherheits,- und Verbraucherschutzgesetz“ (LMSVG).

When it comes to drinking water disinfection, the following procedures are allowed:

- Chlorination with sodium-, potassium-, calcium-, or magnesium hypochlorite
- Chlorination with chlorine gas
- Treatment with chlorine dioxide
- Ozonation
- UV radiation

A residual concentration of free chlorine (Cl_2) should not be lower than 0.3mg/l Cl_2 nor higher than 0.5mg/l Cl_2 after a reaction time of at least 30 minutes when disinfecting with hypochlorite solution or chlorine gas. The end concentration when reaching the consumer should not be more than 0.3 mg/l Cl_2.

Disinfection with at least 0.2mg/l but no more than 0.4 mg/l ClO_2 needs to have a minimum reaction time of 15 minutes whereas one should regard a proper mixture. After the reaction time, a residual concentration of 0.05mg/l ClO_2 has to be detectable. The maximum allowed concentration of the byproduct chlorite when reaching the consumer is 0.2mg/l.

The water quality investigation can be arranged in

- Microbiological (bacteriological) inspection
- Physical and chemical inspection
- Microscopic inspection
- Inspection of radioactivity

For this study, the microbiological investigation is of most relevance with examining the following indicator parameters: colony forming units at 22°C and 37°C, which are limited to 100 and 20 colony forming units/ml respectively.

Table 1: Microbiological indicator parameters for not disinfected water according to „Trinkwasserverordnung“.

PARAMETER	AMOUNT	UNIT
Coliforms	0	quantity/100ml
Enterococci	0	
Pseudomonas aeruginosa	0	
Clostridium perfringens	0	

Source: https://www.ris.bka.gv.at/GeltendeFassung.wxe?Abfrage=Bundesnormen&Gesetzesnummer=20001483; accessed on: 07-03-2022.

Table 2: Microbial water quality standards for poultry drinking water.

SOURCE	GOOD	ACCEPTABLE	UNACCEPTABLE
main water supply	< 100 CFU/ml	< 300 CFU/ml	> 300 CFU/ml
total aerobic plate counts	0 CFU/ml	< 1000 CFU/ml	> 1000 CFU/ml
total coliforms	0 CFU/ml	50 CFU/ml	> 50 CFU/ml
fecal coliforms	0 CFU/ml	0 CFU/ml	1 CFU/ml
E. Coli	0 CFU/ml	0 CFU/ml	1 CFU/ml
Pseudomonas	0 CFU/ml	0 CFU/ml	1 CFU/ml

Source: https://en.engormix.com/poultry-industry/articles/poultry-drinking-water-sanitation-t36573.htm; accessed on: 07-03-2022; Abbreviations: CFU = colony forming units.

The "Trinkwasserverordnung" states that *no E. coli, enterococci* or *P. aeruginosa* are allowed to be detected per 100 ml (Table 1). A total plate count of 1000 colony forming units (CFU)/ml or less is regarded as acceptable. If results exceed 10,000 CFU/ml, it is strongly recommended that stringent cleaning measures be implemented. The water system should be disinfected between stoves, and an individual water purification system should be introduced daily. An indicator of a dirty system may be a bad taste or odor, especially if the percentage of pathogenic germs in the total coliform count is > 50 CFU/ml (Table 2) (https://www.thepoultrysite.com/articles/water-identifying-and-correcting-challenges; accessed on: 07-03-2022).

1.5. AIM OF THE STUDY

This study focused on determining the microbiological contamination of poultry drinking water before and after sanitization using culture-based and sequence-based methods. A total of 114 poultry drinking water samples from five slaughterhouses with 15 different poultry operations were analyzed as part of the Feed and Food Quality Safety and Innovation (FFoQSI) Camp Control project. All water samples were investigated with culture-based methods. Hygiene indicator bacteria (aerobic mesophilic counts, *Enterobacteriaceae*) were determined quantitatively. Additionally, *Campylobacter* and *Salmonella* absence or presence were detected after enrichment. Isolates were confirmed by 16S PCR sequencing methods. The effects of contaminated drinking water can be directly linked to the health and performance of poultry operations. The quantitative and qualitative microbiological investigation is important to verify whether the sanitation measures such as the use of ClO_2 in water pipes have an effect.

2. MATERIALS AND METHODS

2.1. DEVICES AND MATERIALS

All used devices and materials for conducting this study including manufacturers are listed in appendix 1.

2.2. MICROBIOLOGICAL CHARACTERIZATION

2.2.1. WATER SAMPLE PREPERATION

In the following study a total of 114 poultry drinking water samples were analyzed coming from 15 different poultry farms delivering their poultry to 5 slaughterhouses. 300 ml of water samples were centrifuged at 4000 rotations per minute (rpm) at 4°C for 30 minutes in a Sorvall Lynx 4000 centrifuge (Thermo Fisher Scientific Inc., Massachusetts, USA). The supernatant was carefully discarded into the original sample flasks afterwards whereas around 15-20 ml have been left in the centrifugation flask. By adding 45 ml of buffered peptone water (BPW) (Biokar Diagnostics; Pantin Cedex, France), 5 ml of the sediment was suspended in clean Cellstar® 50 ml tubes (Greiner-Bio One, Kremsmünster, Austria) which made a total volume of 50 ml. Furthermore, 1:10 dilutions were prepared in 900 µl BPW up to dilution 10^{-5} (Biokar Diagnostics) were prepared (Figure 1).

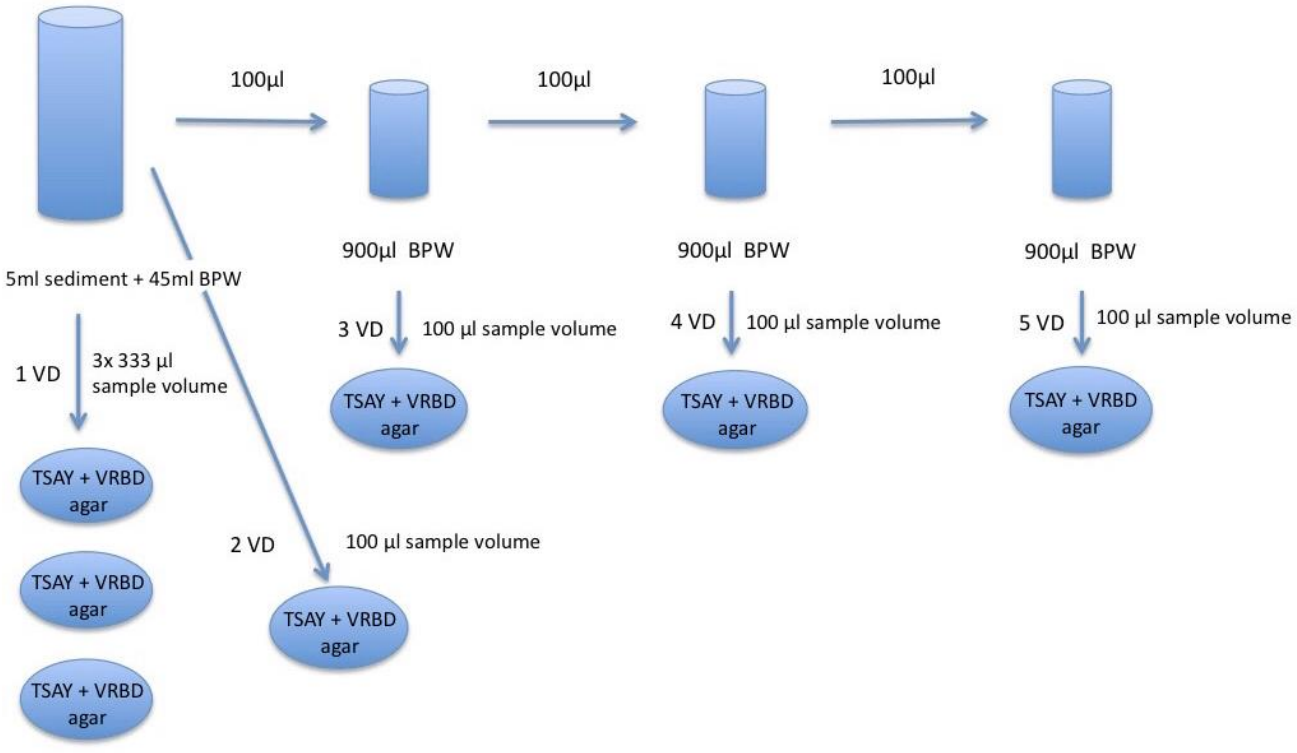

Figure 1: Experimental workflow for the microbiological investigation of hygiene indicator bacteria.

Abbreviations: VD = dilution, BPW = buffered peptone water, TSAY = Trypto-Casein-Soy-Agar, VRBD = Violet-Red-Bile-Dextrose-Agar

Dilutions 10^{-5} (100 µl each) were plated on Trypto-Casein-Soy-Agar (TSAY) (Biokar Diagnostics) and Violet-Red-Bile-Dextrose-Agar (VRBD) (Merck KGaA; Darmstadt, Germany) and incubated at 27°C for 24-48 hours. The BPW suspension (Biokar Diagnostics) was placed at 37°C for 24 hours in order to detect *Salmonella* (see chapter 2.2.7).

2.2.2. PLATE EVALUATION - *ENTEROBACTERIACEAE*

Enterobacteriaceae (target colonies) have the morphology of pink colonies, sometimes surrounded by a precipitation in the agar and *Pseudomonadaceae* (non-target colonies) grow as pale colonies. Therefore, two to three non-target and target colonies were subcultured on TSAY (Biokar Diagnostics) or VRBD (Merck KGaA) agar (see table 3) and incubated at 30°C for 24 to 48 hours. Afterwards, the DNA of each purified isolate was extracted for 16S sequencing. Cryo-stocks were prepared of each isolate and stored at -80°C (see chapter 2.3.2).

Table 3: Compositions of Violet-Red-Bile-Glucose-Agar (VRBG).

Ingredients	Amount
Pancreatic Digest of Gelatin	7 g/l
Yeast Extract	3 g/l
Bile Salts	1.5 g/l
NaCl	5 g/l
Glucose Monohydrate	10 g/l
Neutral Red	30 mg/l
Crystal Violet	2 mg/l
Agar	15 g/l
Water	1000 ml/l
pH at 25°C	7.4 ± 0.2

Source: https://www.merckmillipore.com/AT/de/product/VRBD-Violet-Red-Bile-Dextrose-agar,MDA_CHEM-110275; accessed on: 07-03-2022.

2.2.3. PLATE EVALUATION –AEROBIC MESOPHILIC COUNTS

Colonies grown on TSAY agar (Biokar Diagnostics) were counted and two to three colonies were collected, which were later on subcultured on TSAY agar (Biokar Diagnostics) at 30°C for 24 to 48 hours (see table 4). Afterwards, the DNA of each isolate was extracted for 16S sequencing. Cryo-stocks were prepared of each isolate and stored at -80°C (view chapter 2.3.2).

Table 4: Compositions of Trypto-Casein-Soy-Agar (TSAY).

Ingredients	Amount
Tryptone	15 g/l
Papaic digest of soybean meal	5 g/l
Sodium chloride	5g/l
Bacteriological agar	15 g/l
pH at 25°C	7.3 ± 0.2

Source: https://www.solabia.com/Produto_188,9/BIOKAR-Diagnostics/TRYPTO-CASEIN-SOY-AGAR-TSA-.html; accessed on: 07-03-2022.

2.2.4. PLATE EVALUATION *CAMPYLOBACTER*

Campylobacter colonies grown on Charcoal-Cefoperozone-Deoxycholate-Agar (CCDA) (Thermo Fisher Scientific Inc., Oxoid Ltd.) (Table 5) were evaluated for typical growth. Subsequently, two colonies were collected which were later on subcultured on TSAY agar (Biokar Diagnostics) at 42°C under microaerophilic conditions for 24 to 48 hours. The DNA was extracted with Chelex method of each isolate for 16S sequencing. Cryo stocks in Thermo Scientific™ horse blood laked agar (Thermo Fisher Scientific Inc., Oxoid Ltd.) have been made afterwards.

Table 5: Charcoal-Cefoperazone-Deoxycholate-Agar (CCDA).

Ingredients	Amount
Lab Lemco Powder	10 g/l
Peptone	10 g/l
Sodium Chloride	5 g/l
Bacteriological charcoal	4 g/l
Casein hydrolysate	3 g/l
Sodium desoxycholate	1 g/l
Ferrous sulphate	0.25 g/l
Sodium pyruvate	0.25 g/l
Cefoperazone	0.032 g/l
Amphotericin B	0.01 g/l
Agar	12 g/l
pH at 25°C	7.4 ± 0.2

Source: http://www.oxoid.com/uk/blue/prod_detail/prod_detail.asp?pr=SR0155&c=uk&lang=en; accessed on: 07-03-2022.

2.2.5. BOLTON BROTH SUPPLEMENTED WITH LAKED HORSE BLOOD

To prepare one liter Bolton broth, 27.6 g of Bolton broth agar base (Thermo Fisher Scientific Inc., Oxoid Ltd.) was prepared and 950 ml of demineralized water was added. The medium was autoclaved and cooled at room temperature after heating. Then, 10 ml of modified Bolton broth selective additive (Thermo Fisher Scientific Inc., Oxoid Ltd.) was aseptically added to the sterile Bolton broth. The modified Bolton broth selective additive (Thermo Fisher Scientific Inc., Oxoid Ltd.) was dissolved in ethanol at a 1:1 ratio to sterile distilled water. The bottle was stored at 4°C until further use, and 50 ml of laked horse blood (Thermo Fisher Scientific Inc., Oxoid Ltd.) had to be added before using the Bolton broth for further enrichment.

2.2.6. *CAMPYLOBACTER* CULTIVATION

To determine *Campylobacter* growth, 300 ml of water samples were centrifuged for 30 minutes at 4°C at 4000 rpm in a Sorvall Lynx 4000 centrifuge Thermo Fisher Scientific Inc., Oxoid Ltd.). The supernatant was then carefully discarded into the original sample bottles, while approximately 15 to 20 ml remained in the centrifugation bottle. After this step, 5 ml of the sediment was resuspended in 45 ml of horse blood-enriched Bolton broth (Thermo Fisher Scientific Inc., Oxoid Ltd.) in clean Cellstar® 50 ml tubes (Greiner-Bio One) and then incubated for 48 hours at 42 °C under microaerophilic conditions. After incubation, the Bolton broth enrichment was gently shaken and the CCDA agar (Thermo Fisher Scientific Inc., Oxoid Ltd.) was inoculated by a 10 µl loop (Sarstedt AG & Co. KG, Nürnbrecht, Germany) (Thermo Fisher Scientific Inc., Oxoid Ltd.). CCDA (Thermo Fisher Scientific Inc., Oxoid Ltd.) was incubated for 48 hours at 42 °C under microaerophilic conditions. The microbiological investigation for detecting *Campylobacter* was conducted via the "International Organization for Standardization" (ISO) method 10272-1 (2017).

2.2.7. *SALMONELLA* CULTIVATION

To determine *Salmonella* growth, the BPW suspension (Biokar Diagnostics) was first incubated at 37°C for 24 hours. The next day, 1 ml of the enrichment was transferred to Muller-Kaufmann Tetrathionate Novobiocin (MKTTn) Broth (Biokar Diagnostics) and incubated for 24 hours at 37°C. In the next step, 100 µl of BPW suspension (Biokar Diagnostics) was transferred to Rappaport-Vassiliadis soy peptone broth (RVS) (Thermo Fisher Scientific Inc., Oxoid Ltd.) and incubated at 42°C for 24 hours. MKKTn (Biokar Diagnostics) and RVS (Thermo Fisher Scientific Inc., Oxoid Ltd.) enrichments were plated on xylose-lysine-deoxycholate agar (XLD) (Thermo Fisher Scientific Inc., Oxoid Ltd) plates and incubated at 37°C for 24-48 hours. In case of *Salmonella*-specific growth, DNA was extracted, 1 to 2 colonies were subcultured, and cryo-stocks were prepared.

For determining *Salmonella* growth, the BPW suspension (Biokar Diagnostics), was first placed at 37°C for 24h. On the next day, 1 ml of the enrichment was transferred to Bouillon Muller-Kaufmann Tetrathionate Novobiocin (MKTTn) (Biokar Diagnostics), and incubated at 37°C for 24h. As a next step, 100 µl of the BPW suspension (Biokar Diagnostics), were transferred to RVS (Thermo Fisher Scientific Inc., Oxoid Ltd.) and incubated at 42°C for 24h. The MKKTn (Biokar Diagnostics) and RVS enrichments (Thermo Fisher Scientific Inc., Oxoid Ltd.) were plated on Xylose-Lysine-Desoxycholate-Agar (XLD) (Thermo Fisher Scientific Inc., Oxoid Ltd.) plates and incubated at 37°C for 24-48 hours. In case of *Salmonella* specific growth, the DNA was extracted, 1 to 2 colonies subcultured and cryo stocks were made. The microbiological investigation for detecting *Salmonella* was conducted via ISO method 6579-1 (2017).

2.3. MOLECULARBIOLOGICAL CHARACTERISATION

2.3.1. CHELEX DNA EXTRACTION

For producing 100 ml of Chelex solution, 95 ml bidistilled water using Merck Millipore Milli-Q™ Reference Ultrapure Water Purification System (Merck KGaA) 2.5 g Chelex Resin® (Bio-Rad Laboratories Inc., Hercules, California), 2.5 ml 0.01M Trisaminomethane (Tris) HCL pH 7 (Merck KGaA) were used and autoclaved afterwards. In order to extract DNA, the heating block (Kleinfeld Labortechnik GmbH., Gehrden, Germany) needed to be preheated at 100°C. Maxymum recovery tubes (Axygen Inc., California, USA) were labeled with each isolate number. One extra Maxymum recovery tube (Axygen Inc.) was taken for negative control. Afterwards, 100 µl of 0.01M Tris/HCL (Merck KGaA) pH 7 were pipetted into safe-lock tubes (Eppendorf AG, Hamburg, Germany). Bacterial material was collected with blue 10 µl inoculation loops (Sarstedt AG & Co. KG) and resuspended in 2 ml safe-lock tubes (Eppendorf AG, Hamburg, Germany) with 0.01M Tris/HCl (Merck KGaA) tubes. 400 µl of Chelex solution was added, vortexed afterwards and placed on the 100°C heating block (Kleinfeld Labortechnik GmbH.) for 10 minutes. After the time has passed, the 2ml safe-lock tubes (Eppendorf AG) were centrifuged in the centrifuge 5424 (Eppendorf AG, Hamburg, Germany) at 15000 rcf for 5 seconds. 100 µl of the supernatant was pipetted into the Maxymum recovery tubes (Axygen Inc.) and stored at -20°C in an appropriate box until further use.

2.3.2. CRYO-STOCKS

Cryo stocks were prepared aseptically in order to store the isolate set appropriately at -80°C. For one liter of cryo- stock solution 750 ml Brain Heart Broth (BHI) medium (Biokar Diagnostics) and 250 ml 60% glycerol (Merck KGaA) were mixed. Cryogenic vials (Biologix Group Ltd., Shandong, China) were labeled and filled with 1 ml of cryo stock solution each. Bacterial material was collected with blue 10

µl inoculation loops (Sarstedt AG & Co. KG) and resuspended in BHI plus Glycerol and stored in cryogenic vials (Biologix Group Ltd.).

2.3.3. 16S rRNA SEQUENCING

For further species confirmation of the isolate collection, 16S rRNA PCR was performed using universal primers 616F and 1492R according to the protocol of JURETSCHKO et al. (1998) and Lane (1991) and then sent to LGC Genomics (Berlin, Germany) for sequencing.

The PCR mastermix and cycler conditions can be obtained from Table 6. The DNA was diluted 1:100 with 990 µl 0.01M Tris/HCl (Merck KGaA) and 10 µl of the DNA.

Table 6: Mastermix for 16S PCR sequencing.

Mastermix	Final concentration	unit	Stock concentration	unit	1x	35x
DEPC water					31.1	1088.5
10x PCR buffer	1x				5	175
MgCl2	2	mM	50	mM	2	70
616F	200	nM	5000	nM	2	70
1492R	200	nM	5000	nM	2	70
dnTP's	250	µM	5000	µM	2.5	87.5
Taq Pol (Plat)	2	U	5	U/µl	0.4	14
Mastermix					40	1575
Template					5	
Reaction Volume					45	

Source: JURETSCHKO et al. (1998), LANE (1991).

Table 7: Cycler conditions for 16S PCR sequencing.

PCR conditions	Temperature	Duration	Number of cycles
Initial denaturation	95°C	5 minutes	
Denaturation	94°C	30 seconds	35 cycles
Annealing	52°C	30 seconds	
Elongation	72°C	60 seconds	

Final elongation	72°C	7 minutes	
Storage	4°C	hold	

Source: JURETSCHKO et al. (1998), LANE (1991).

PCR product quality was evaluated in a 1.5% agarose gel prepared by melting 1.5 g agarose (VWR International GmbH, Darmstadt, Germany) in 100 ml trisaminomethane acetate-ethylenediaminetetraacetic acid (TAE) buffer (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) in a microwave (Schneider GmbH, Salzburg, Salzburg). After addition of 2 µl PEQ Green (VWR International GmbH, Darmstadt, Germany), the gels were cast and cooled in 20 slits until cured. The first and last slot of each row was filled with 8 µl Thermo Scientific™ GeneRuler™ 1kb DNA ladder (Thermo Fisher Scientific Inc.). Thereof, 3 µl of sample loading buffer (Thermo Fisher Scientific Inc.,) and 5 µl of DNA were added to each of the remaining slots. The gel was run at 120 V for 30 min and then analyzed using GELDOC 2000. The results were documented in Tiff format.

The PCR amplicons were sent to LGC Genomics for SANGER sequencing (https://shop.lgcgenomics.com/; accessed on: 07-03-2022).

The sequences were converted in a nucleotide basic local alignment search tool (BLAST) and compared to international sequencing data banks.

BLAST finds similarities between biological sequences. The program compares nucleotides or protein sequences with sequence data banks and calculates the statistical significance (https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed on: 07-03-2022).

The rRNA/IST databases option was chosen and the option highly similar sequences (megablast) was selected. In the results, the sequence information for the current isolates were shown. For each result the description, maximum score, total score query cover, E value, percent identity and accession were noted.

3. RESULTS

3.1. SAMPLES

The 114 investigated water samples originated from 15 broiler farms (farm code in numbers) associated to five slaughterhouses (A-E) (Figure 2 A). Thereof, 69 (n=69/114; 60.5%) and 45 (n=45/114; 39.5%) water samples were collected before and after sanitation (Figure 2B). The majority of water samples were taken at broiler farm 40 (n=16/114; 14.04% of samples; delivering to abattoir A), broiler farm 24 (n=13/114; 11.4% of samples; abattoir B), and broiler farm 41 (n=12/114; 10.53%; abattoir C) (Figure 2C).

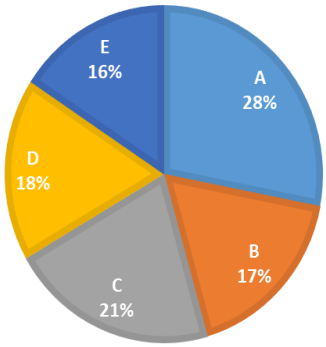

Figure 2A: Percentage of water samples assigned to slaughterhouses A-E.

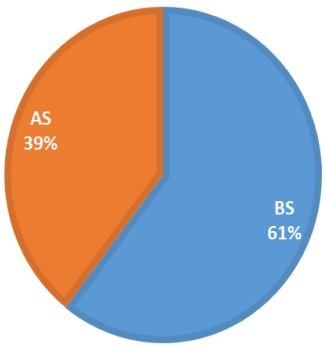

Figure 2B: Percentage of water samples before und after sanitation.
Abbreviations: AS = after sanitation, BS = before sanitation.

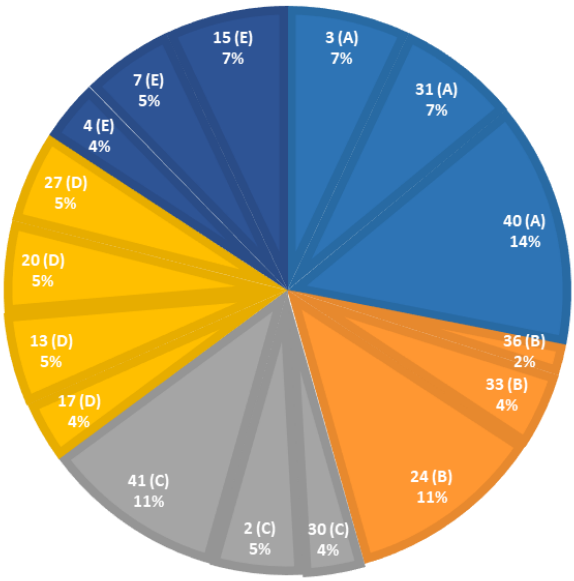

Figure 2C: Percentage of water samples assigned to slaughterhouses (A-F) and broiler farms (numbers).

Figure 2 A-C: Distribution of water samples associated to slaughterhouses and broiler farms.

The aerobic mesophilic count (AMC), *Enterobacteriaceae* (EB) and *Pseudomonadaceae* (PS) counts were determined before and after sanitation of each water sample and are depicted in Figure 3. The majority of samples exceeded AMC counts before and after sanitation in the range of 4.0-5.9 $\log_{10}$ cfu/ml (n=26/69; 37.7% and n=16/45; 35.6% of samples). AMC counts ≥6 $\log_{10}$ cfu were more likely to be detected prior to sanitation (n=20/69; 29.0% versus n=4/45; 8.9% of samples). The EB counts exceeded >4.0-5.9 $\log_{10}$ and ≥6 $\log_{10}$ cfu for n=9/69; 13.0% and n=2/69; 2.9% samples before and n=3/45; 6.7% samples after sanitation (>4.0-5.9 $\log_{10}$/ml). A higher amount of PS count was observed in n=29/69; 42.0% and n=13/45; 28.9% of samples before and after sanitation within the range (>4.0-5.9 $\log_{10}$/ml). In general, a bacterial reduction was evident for the majority of samples after sanitation, but the critical limit of <10,000 cfu/ml (>4.0-$\log_{10}$/ml) poultry drinking water was still not achieved for some hygiene indicators (Figure 3).

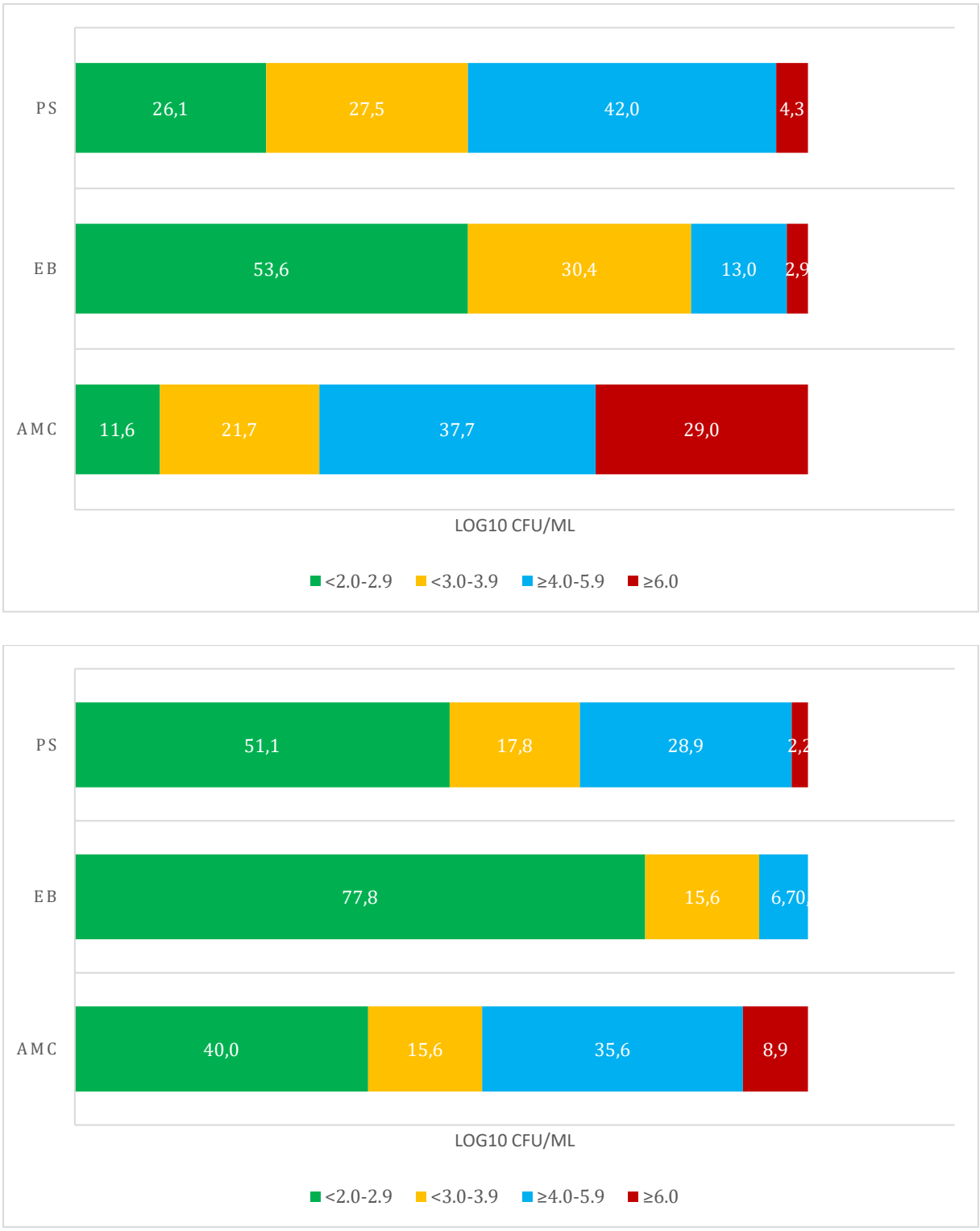

Figure 2: Percentages of the bacterial count before (top) and after (bottom) sanitation.

Abbreviations: CFU = colony forming units.PS = *Pseudomonadaceae.* EB = *Enterobacteriaceae*, AMC = total aerobic cell count.

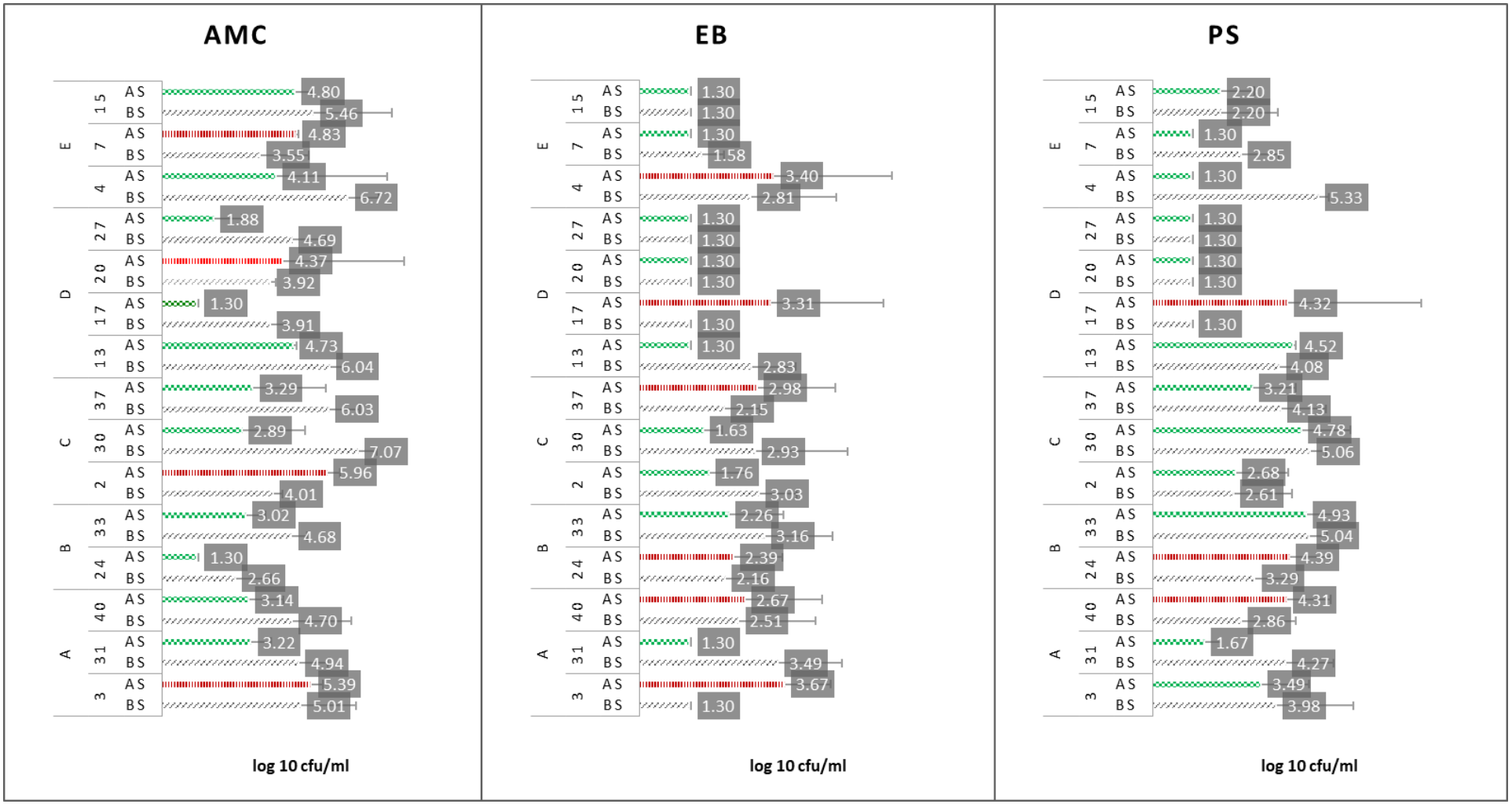

Figure 3: Comparison of the after sanitation and before sanitation effect on broiler farm level.

Abbreviations: CFU = colony forming units.PS = *Pseudomonadaceae.* EB = *Enterobacteriaceae*, AMC = total aerobic cell count, AS = after sanitation, BS = before sanitation.
Color coding red shows no log reduction after sanitation, green color code indicates a log reduction after sanitation.

When comparing the water hygiene in the individual poultry farms, there was no clear log reduction of bacterial counts at all individual farms observed after the cleaning measurements of the drinking line (Figure 4). In detail, there was no AMC log reduction achieved after sanitation at broiler farm 3 (assigned to abattoir A), broiler farm 2 (abattoir C), broiler farm 20 (abattoir D) and broiler farm 7 (abattoir E) (all counts >4.0 log_{10} cfu/ml).

EB counts were higher after sanitation at broiler farm 3 and 40 (assigned to abattoir A), broiler farm 24 (abattoir B), broiler farm 37 (abattoir C), broiler farm 17 (abattoir D) and broiler farm 4 (abattoir E). PS counts were increased after sanitation in broiler farm 40 (abattoir A), broiler farm 24 (abattoir B) and broiler farm 17 (abattoir D) (all counts >4.0 log_{10} cfu/ml).

In conclusion AMC and EB counts were increasing after sanitation in broiler farm 3 and 7 (abattoir A and E) and EB and PS counts were increasing in water samples of broiler farm 40, 24 and 17 (abattoir A, B and D) (Table 8).

Table 8: Comparison of broiler farms exceeding values >10,000 cfu/ml with a lack in waterline sanitation efficiency.

BROILER FARM CODE			**ABATTOIR CODE**
AMC	**EB**	**PS**	
3	**3**		A
	40	**40**	A
	24	**24**	B
2			C
	37		C
20			D
	17	**17**	D
7	**7**		E

Marginal AMC log reduction after sanitation was recorded in farm 13 (abattoir D), 4 and 15 (abattoir E), where levels still exceeded >10,000 cfu/ml poultry drinking water (Figure 4).

3.2. BACTERIAL ISOLATE CHARACTERISTICS

About 395 bacterial isolates from water samples before (n=272/395; 68.9%) and after sanitation (n=123/395; 31.1%) of poultry drinking water pipelines were assigned to bacterial phyla (Figure 5) and families (Figure 6).

Proteobacteria formed the majority of isolates (n=322/395; 82%), followed by Firmicutes (n=54/395; 14%), Bacteriodetes (n=12/395; 3%) and Actinobacteria (n=6/395; 1%). When comparing families, *Pseudomonadaceae* (n=123/395; 31 %), *Enterobacteriaceae* (n=59/395; 15 %) and *Comamonadaceae* (n=54/395; 14 %) were the most frequently isolated bacterial families (Figure 6).

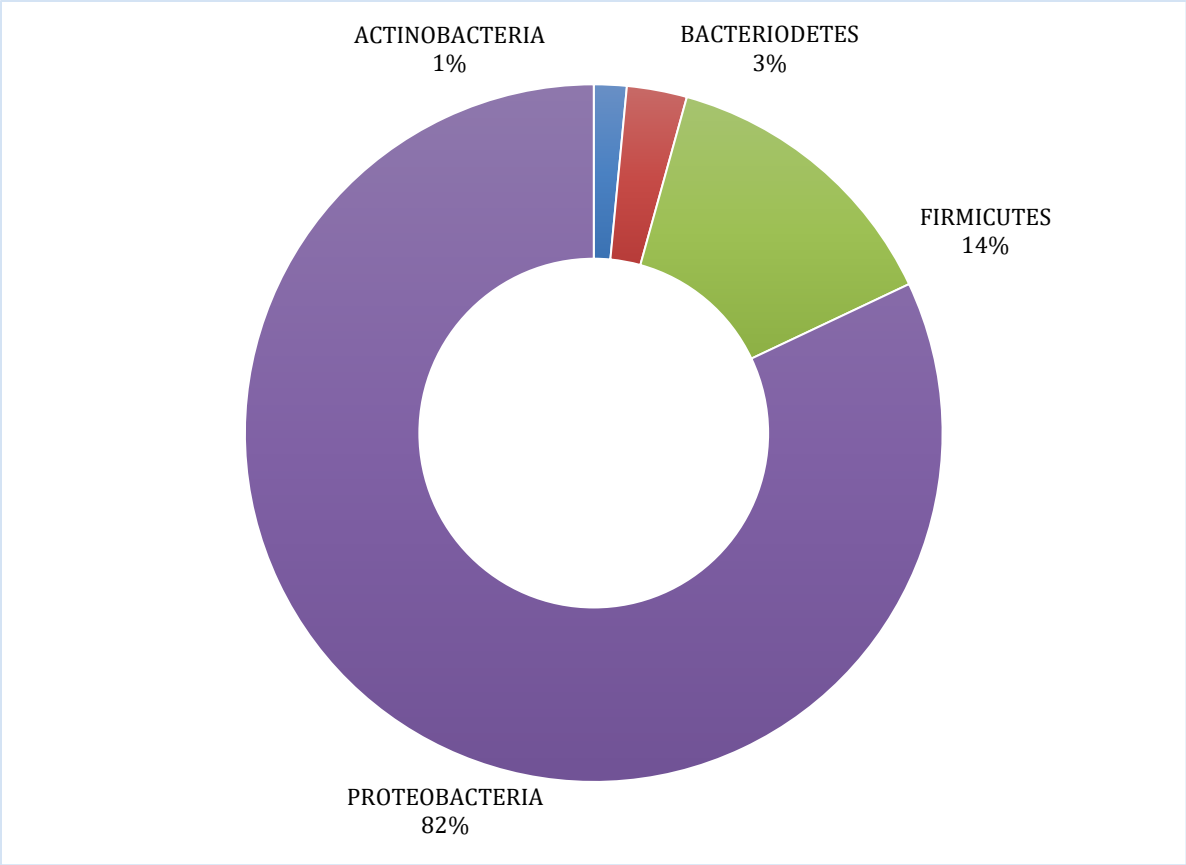

Figure 4: Bacterial isolates originating from poultry drinking water samples assigned to phyla.

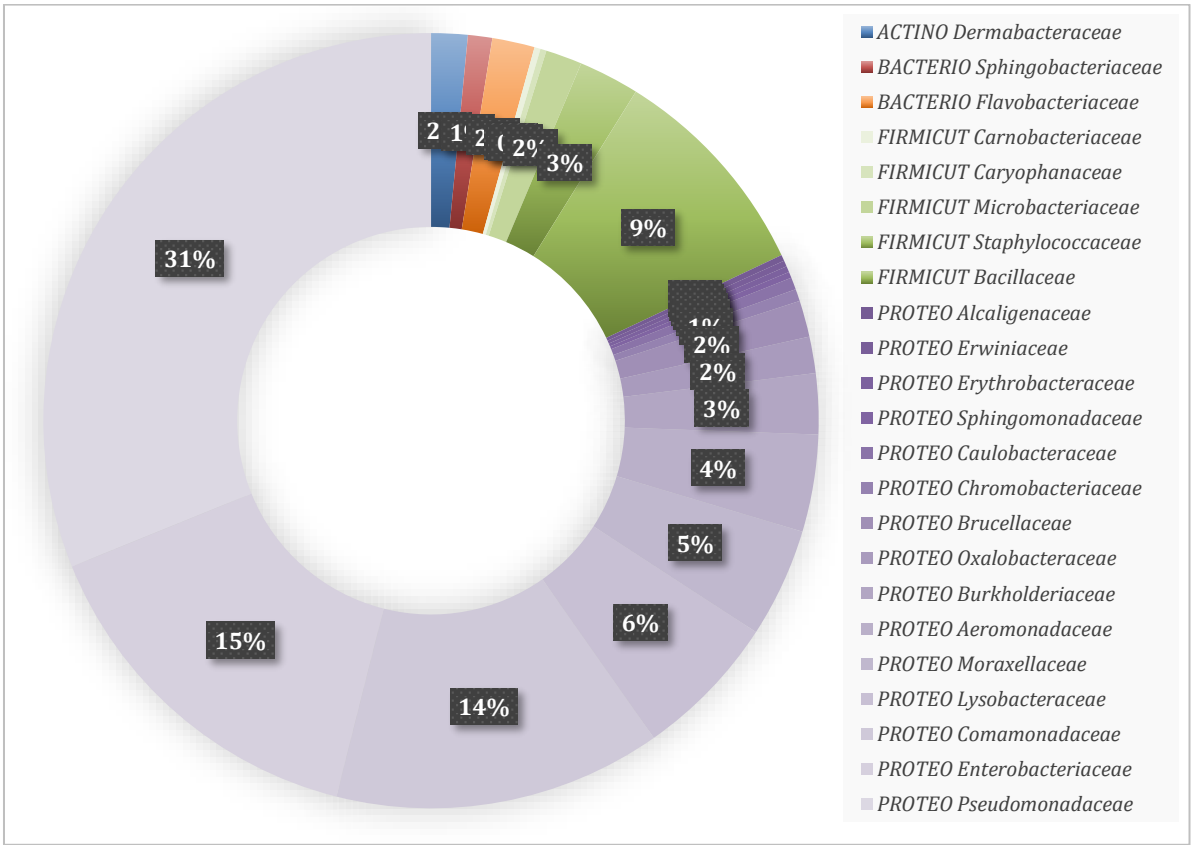

Figure 5: Bacterial isolates assigned to phyla and families.

Abbreviations: PROTEO = Proteobacteria, ACTINO = Actinobacteria, FIRMICUT = Firmicutes.

The most abundant genera before and after sanitation analyzed on the base of isolates is depicted in Table 9. The genera that were present either only before or exclusively after cleaning are written in bold (Table 9). About 25 and 20 different genera were detected before after sanitation among the group of Proteobacteria, respectively. In the phylogenetic group of Firmicutes (second most abundant phylum), the number of genera was reduced from six to three after sanitation, with *Bacillus* and *Neobacillus* still present and *Solibacillus* as newly isolated genus. *Brachybacterium* (Actinobacteria) was only present before sanitation.

Table 9: Comparison of bacterial isolates on the base of phyla, families and genera before and after sanitation.

Category	Phylum	Familiy	Genus	n	Category	Phylum	Familiy	Genus	n
BS	PROTEO	Pseudomonadaceae	Pseudomonas	88	AS	PROTEO	Pseudomonadaceae	Pseudomonas	35
		Lysobacteraceae	Stenotrophomonas	16			Lysobacteraceae	Stenotrophomonas	8
		Comamonadaceae	Comamonas	15			Comamonadaceae	Comamonas	5
			Variovorax	12				Variovorax	2
			Acidovorax	4				Acidovorax	11
			Delftia	3					
			Hydrogenophaga	1				Hydrogenophaga	1
		Enterobacteriaceae	Citrobacter	13			Enterobacteriaceae	Citrobacter	7
			Klebsiella	8				Klebsiella	1
			Buttiauxella	2					
			Enterobacter	4				Enterobacter	8
			Escherichia	3				Escherichia	1
			Raoultella	5					
			Leclercia	2					
			Phytobacter	2					
								Kluyvera	1
			Lelliottia	1				Lelliottia	1
		Erythrobacteraceae	Novosphingobium	1					
		Aeromonadaceae	Aeromonas	11			Aeromonadaceae	Aeromonas	4
			Pseudaeromonas	1					
		Burkholderiaceae	**Cupriavidus**	10					
		Moraxellaceae	Acinetobacter	8			Moraxellaceae	Acinetobacter	8
								Moraxella	2
		Brucellaceae	Ochrobactrum	5			Brucellaceae	Ochrobactrum	1
		Caulobacteraceae	Brevundimonas	1			Caulobacteraceae	Brevundimonas	1
		Chromobacteriaceae	**Chromobacterium**	2					
							Oxalobacteraceae	**Herminiimonas**	3
								Janthinobacterium	3
							Erwiniaceae	Pantoea	1
		Alcaligenaceae	**Pigmentiphaga**	1					
	ACTINO	Dermabacteraceae	**Brachybacterium**	6		ACTINO			
	BACTERIO	Flavobacteriaceae	Flavobacterium	4		BACTERIO	Flavobacteriaceae	Flavobacterium	3
		Sphingobacteriaceae	**Sphingobacterium**	3					
			Pedobacter	2					
	FIRMICUT	Bacillaceae	Bacillus	19		FIRMICUT	Bacillaceae	Bacillus	12
			Neobacillus	2				Neobacillus	3
		Staphylococcaceae	**Staphylococcus**	9					
			Jeotgalicoccus	1					
		Microbacteriaceae	**Microbacterium**	6					
							Caryophanaceae	**Solibacillus**	1
		Carnobacteriaceae	**Trichococcus**	1					
Total				**272**	**Total**				**123**

Abbreviations: BS = before sanitation, AS = after sanitations.

Table 10: Most abundant species on sample and broiler farm level.

	Abattoir	A			B			C				D				E		
	Broiler farm	**3**	**31**	**40**	**24**	**33**	**36**	**2**	**20**	**30**	**37**	**13**	**17**	**20**	**27**	**4**	**7**	**15**
PROTEO	*P. aeruginosa*		BS&AS	BS		BS		AS		AS	BS							
	P. taiwanensis	AS		BS						BS	BS							
	P. veronii			BS	BS				BS	BS			AS			BS		
	P. resinovorans									BS	BS						AS	
	P. proteolytica		BS					AS										
	P. brassicacearum subsp. neoaurantiaca			AS	BS													
	P. rhodesiae			BS														AS
	P. putida		BS	BS		BS				BS	BS							BS
	P. koreensis			BS	BS							BS						
	Stenotrophomonas maltophilia			BS		BS		AS			BS				BS	BS	BS	AS
	Comamonas testosteroni	AS		BS				BS &AS						BS				
	Acidovorax temperans					AS					AS							
	Variovorax boronicumulans		BS	BS&AS		AS						BS						
	Variovorax paradoxus										BS	BS			BS	BS		
	Citrobacter murliniae							BS	BS							AS	BS	
	Citrobacter europaeus							BS								AS	BS	BS
	Citrobacter freundii	AS							BS			AS		AS	BS			
	Klebsiella grimontii															AS		BS
	Enterobacter hormaechei subsp. xiangfangensis		AS		BS	BS				BS		AS						
	Enterobacter ludwigii																BS&AS	
	Aeromonas salmonicida	BS	BS									AS						
	Aeromonas rivipollensis	AS	BS									BS						
	Aeromonas hydrophila							AS			BS							
	Cupriavidus campinensis			BS			BS											
	Cupriavidus metallidurans										BS		BS		BS			
	Acinetobacter radioresistens	BS		BS				AS										
	Acinetobacter lwoffii			BS				AS				BS						
	Acinetobacter johnsonii										BS				BS			AS
	Ochrobactrum intermedium	BS&AS	BS	BS														
ACTINO	*Brachybacterium paraconglomeratum*			BS						BS		BS						
FIRMICUT	*BSG*	BS&AS		BS &AS			BS					BS&AS	BS		BS&AS		BS	BS&AS
	Bacillus bataviensis	BS&AS			AS													

Abbreviations: BS = before sanitation, AS = after sanitation; BSG =*Bacillus subtilis* group (*Bacillus nakamurai, Bacillus velezensis, Bacillus licheniformis, Bacillus tequilensis, Bacillus halotolerans*); PROTEO = Proteobacteria; ACTINO = Actinobacteria; FIRMICUT = Firmicutes.

In the group of Bacteriodetes, three genera, including *Flavobacterium*, were detected before cleaning; *Flavobacterium* was still detected after cleaning. *Pseudomonas* comprised the majority of isolates before (n=88) and after sanitation (n=35), followed by *Stenotrophomonas* (n=16 before sanitation-BS, n=8 after sanitation-AS), *Bacillus* (n=19 BS and n=12 AS), *Comamonas* (n=15 BS and n=5 AS), *Citrobacter* (n=13 BS and n=7 AS) and *Acinetobacter* (n=8 each BS and AS).

The most relevant bacterial species isolated before and after sanitation of distinct broiler farms (broiler farm 3, 31 and 40, abattoir A) were *Pseudomonas aeruginosa,* a risk group 2 organism and *Comamonas testosteroni, Variovorax boronicumulans, Bacillus subtilis* group and *Bacillus bataviensis*, all assigned to risk group 1 (https://bacdive.dsmz.de/; accessed on: 07-03-2022).

Ochrobactrum (Brucella) intermedium was present in broiler farm 2 (abattoir C) before and after sanitation and *Enterobacter ludwigii* (risk group 2) in broiler farm 7 (abattoir E). *Bacillus subtilis* group was also present before and after sanitation in farm 13 and 27 (abattoir D) (Table 10).

4. DISCUSSION AND CONCLUSION

This study focused at drinking water quality and safety in Austrian broiler farms. As part of the Feed and Food Quality Safety and Innovation (FFoQSI) research project, a project was launched with industry partners to reduce and prevent *Campylobacter* spp. in primary production as part of "Camp Control". A large part of these measures concerned the individual levels of biosecurity on poultry farms. These included the functionality of the hygiene sluice, broiler house cleaning and disinfection before new broiler flocks arrived, litter quality and also drinking water hygiene and quality. The latter biosecurity criterion focused on the cleanability of the water pipes and nipple drinkers. The standard was that the pipes were only thoroughly cleaned before occupation of the barn with a new broiler herd. The pipes were flushed with drinking water irregularly and often, especially when the water pipelines were blocked by biofilms. A constant addition of chlorine dioxide, as tried in other countries, did not take place in any of the test farms before the start of the study (https://www.poultryworld.net/Health/Partner/2021/3/Alternative-water-disinfection-methods-during-production-726760E/; accessed on: 07-03-2022). In selecting suitable broiler farms, consideration was given to those that did not satisfactorily meet all biosecurity requirements due to their structural conditions, etc.

Constant drinking water disinfection with chlorine dioxide was not considered realistic in this study, as it would affect the water intake of the broilers. In detail, the poultry farmers explained that the broilers are very sensitive to a bitter taste caused by chlorine or hypochlorite in the drinking water and would neglect the important source of water intake, which is directly related to health and weight gain (https://www.thepoultrysite.com/articles/water-identifying-and-correcting-challenges; accessed on: 07-03-2022).

Drinking water is a very sensitive commodity in the poultry sector, as medicines, vitamins and probiotics are applied via water during fattening. For this reason, the drinking water line should always contain drinking water quality, which is more than difficult to achieve, as the additives have the property of depositing in the line and favoring the formation of biofilms. Cleaning in Place (CIP) cleaning of the milking system is a common practice in the dairy sector, but not yet state of the art in poultry production (https://farmwatersystems.com/cip/; accessed on: 07-03-2022). In general, poultry drinking water should meet the quality parameters of the Drinking Water Ordinance, which could not be achieved in the majority of water samples collected at the end of poultry water lines (AMC <2 log n=3/15 broiler farms; see Figure 3) (https://www.ris.bka.gv.at/GeltendeFassung.wxe?Abfrage=Bundesnormen&Gesetzesnummer=20001483; accessed on: 07-03-2022). According to literature and guidelines, the limit for AMC in poultry drinking water systems is 3.0 to 5.0 log cfu/ml and the limit for coliform bacteria is 50-100 cfu/ml (http://extension.msstate.edu/sites/default/files/publications/publications/P2754_web.pdf; https://www.biomin.net/science-hub/how-to-improve-poultry-drinking-water-quality-with-acidification/; https://afs.ca.uky.edu/files/chapter12.pdf; accessed on: 07-03-2022). The AMC counts in poultry drinking water samples was exceeding 4.0 log cfu in 11/15 and 5/15 broiler farms before and after sanitation. The number of *Enterobacteriaceae* exceeded 2.0 log/cfu in water samples collected from 9/15 and 7/15 broiler farms before and after cleaning. *Pseudomonadaceae* values exceeded 3.0 log cfu/ ml drinking water at 8/15 and 7/15 broiler farms before and after sanitation (Figure 4). These higher microbiological values quantified in our study (Figure 3 and 4) are consistent with the observations of MAES et al. (2019), who studied water quality after disinfection, where AMC values were still 6.0 log/20cm^2. Because chicken farms tend to flush their pipelines without mechanical pumps, the pipelines tend to become clogged with anorganic and organic material forming the basis for biofilms and resulting in a higher AMC (GOMES et al., 2018).

The most dominant bacterial genera before and after water sanitation were *Pseudomonas, Stenotrophomonas, Bacillus, Comamonas, Citrobacter* and *Acinetobacter*. Accordingly, the water samples were dominated by gram-negative bacteria, except for *Bacillus*, which is consistent with literature (MAES et al., 2019; MOHAMMED et al., 2020). *Firmicutes, Bacteroidetes, Proteobacteria* and *Actinobacteria* are statistically the most common phyla in the gastrointestinal tract (GIT) of poultry which would correlate to this current study suggesting there is an interchange between the water pipe line and GIT (WAN et al., 2021).

Pseudomonas aeruginosa appeared to be very common among poultry water samples, which is significant because it is a water-affinity opportunistic pathogen responsible for biofilm formation. *Pseudomonas aeruginosa* is most abundant in primary production, water, and soil (WEI et al., 2020).

RIBEIRO et al. (2014) followed drinking water produced from karstic regions and detected *Pseudomonas* predominantly from springs to the water tap. *Pseudomonas* is very resistant to stressors in the aquatic environment, thus some strains show increased tolerance or resistance to antibiotics and disinfectants (RIBEIRO et al., 2014; HU et al., 2021; WEI et al., 2020).

Antibiotic resistance can be acquired and persist in several *Pseudomonas* species, which represent a reservoir of resistance genes that can be transferred to multiple pathogenic bacteria by horizontal gene transfer (KITTINGER et al., 2016). Sessile bacteria (e.g. *Pseudomonas* or *Stenotrophomonas*) in water biofilms are monitored for the spread of antibiotic and disinfectant resistance triggered by emerging contaminants (GOMES et al., 2018; https://www.sciencedirect.com/topics/earth-and-planetary-sciences/emerging-contaminant; accessed on: 07-03-2022). Furthermore, *Stenotrophomonas maltophila* played an important role as an opportunistic pathogen organism in biofilm formations. *S. maltophila* produces flagella and attaches to abiotic and living exteriors (DI BONAVENTURA et al., 2004). *Variovorax boronicumulans* is a *Rhizobacterium,* which has a positive influence on plant growth and is strongly associated with the plant-water interface (SUN et al., 2017). WAN et al. (2021) studied the microbial diversity in water pipes from layer hen houses and found *Acinetobacter* and *Comamonas*, among others, to be the most common genera in poor quality drinking water, which is comparable to our data. *C. testosteroni* is a bacterium commonly found in biofilms in bioreactors for the treatment of domestic and industrial wastewater (WU et al., 2015). *Ochrobactrum intermedium* (present in broiler farm 2) and closely related to *Brucella*, are considered to be of low virulence, still they are related as opportunistic pathogens to human infections (RYAN and PEMBROKE, 2020).

Staphylococcus, Enterococci, Sphingopyxis, Bacillus and *Acinetobacter* were detected in poultry drinking water heavily contaminated with antibiotics (ABOELSEOUD et al., 2021).

Bacillus bataviensis and *Bacillus subtilis* group were isolated in poultry water samples investigated in this study. The common usage of chlorine disinfectants for sanitation may cause chlorine resistant spores shown in *Bacillus spp.* (DING et al., 2019). Disinfectants like chlorine, water age and pipe material have shown to be strong factors in biofilm formation (DOUTERELO et al., 2016). Residual chlorine seemed to be highly ineffective as its residue favored bacterial development even more (WANG et al., 2014). HEINEMANN et al. (2020) traced back the entry of pathogenic bacteria with resistance potential in broiler farms with health problems. *Enterobacteriaceae* were already present in the first days of hatching and colonized the chicks. Antibiotic resistance of opportunistic pathogenic bacteria spread during rearing and fattening as antibiotic substances were increasingly applied. Therefore, monitoring of drinking water quality and the presence of hygiene indicators (e.g. *Enterobacter* or *Pseudomonas)* and resulting measures to improve water quality would improve the health status of chicks and broilers.

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6. ACKNOWLEDGEMENT

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7. EXTENDED SUMMARY

Ensuring high-quality poultry drinking water is of great importance for a well-functioning herd performance. Therefore, disinfectants such as sodium hypochlorite, chlorine gas, and calcium hypochlorite are widely used in poultry farms to disinfect drinking water. As part of the Feed and Food Quality Safety and Innovation (FFoQSI) Camp Control project, a total of 114 poultry drinking water samples from five slaughterhouses with 15 assigned poultry farms were microbiologically tested. The cleaning and disinfection of the drinking line includes a basic cleaning with peracetic acid, followed by disinfection with chlorine dioxide. The cleaning was supported mechanically with a pump. The aim of this study was to compare the microbiological contamination of poultry drinking water before and after disinfection using culture-based methods and subsequent 16-S sequencing of the isolate set. The total mesophilic bacterial count, *Enterobacteriaceae* and *Pseudomonadaceae* count were determined quantitatively. From 114 water samples from 15 broiler farms, a total of 69 (n=69/114; 60.5%) and 45 (n=45/114; 39.5%) were taken before and after disinfection. In general, bacterial reduction was evident in the majority of samples after disinfection, but not in all aspects. The size of the mesophilic total bacterial count decreased from 20 out of 69 samples before disinfection to 4 out of 45 samples after disinfection, the number of *Enterobacteriaceae* from 9 out of 69 before disinfection (VD) to 3 out of 45 after disinfection (ND) and *Pseudomonadaceae* from 29 to 69 (VD) to 13 out of 45 (ND). The most frequently isolated bacterial families were *Pseudomonadaceae* (n=123/395; 31%), *Enterobacteriaceae* (n=59/395; 15%) and *Comamonadaceae* (n=54/395; 14%). It was evident that *Pseudomonadaceae* were present in the majority of isolates before (n=88) and after disinfection (n=35) followed by *Stenotrophomonas* (n=16 VD, n=8 ND), Bacillus (n=19 VD and n= 12 ND), *Comamonas* (n=15 VD and n=5 ND), *Citrobacter* (n=13 VD and n=7 ND) and *Acinetobacter* (n=8 each VD and ND). The most relevant bacterial species isolated before and after disinfection were *Pseudomonas aeruginosa*, which belong to risk group 2, and *Comamonas testosteroni, Variovorax boronicumulans, Bacillus subtilis* group and *Bacillus bataviensis*, all of which belong to risk group 1. In summary, water disinfection did indeed reduce the bacterial load, but not in all broiler farms, and there was no significant logarithmic increase in bacterial counts in all individual farms performed after drinking line disinfection.

8. ZUSAMMENFASSUNG

Die Sicherstellung von qualitativ hochwertigem Geflügel-Trinkwasser ist für eine gut funktionierende Herdenleistung von großer Bedeutung. Daher werden Desinfektionsmittel wie Natriumhypochlorit, Chlorgas und Calciumhypochlorit häufig in Geflügelfarmen verwendet, um Trinkwasser zu desinfizieren. Im Rahmen des Projekts Feed and Food Quality Safety and Innovation (FFoQSI) Camp Control wurden insgesamt 114 Geflügel-Trinkwasserproben aus fünf Schlachthöfen mit 15 zugeordneten Geflügelbetrieben mikrobiologisch untersucht. Die Reinigung und Desinfektion der Tränkeleitung umfasste eine Grundreinigung mit Peressigsäure, anschließende Desinfektion mit Chlordioxid. Die Reinigung wurde mechanisch mit einer Pumpe unterstützt,

Ziel dieser Studie war es, die mikrobiologische Kontamination von Geflügeltrinkwasser vor und nach der Desinfektion mit kulturbasierten Methoden und anschließender 16-S Sequenzierung des Isolatsets zu vergleichen. Die mesophile Gesamtkeimzahl, *Enterobacteriaceae u*nd *Pseudomonadaceae* Zahl wurden quantitativ eruiert. Von 114 Wasserproben aus 15 Mastbetrieben wurden insgesamt 69 (n=69/114; 60,5 %) und 45 (n=45/114; 39,5 %) vor und nach der Desinfektion entnommen. Im Allgemeinen war bei der Mehrheit der Proben nach der Desinfektion eine Bakterienreduktion offensichtlich, jedoch nicht in allen Aspekten. Die Größe der mesophilen Gesamtkeimzahl sank von 29,0 % (n=20/69) vor der Desinfektion auf 8,9 % (n=4/45) nach der Desinfektion, die Zahl der *Enterobacteriaceae* von 13,0 % (n=9/69 vor Desinfektion-VD) auf 6,7 % (n=3/45 nach Desinfektion ND) und *Pseudomonadaceae* von 42,0 % (n=29/69 VD) auf 28,9 % (n=13/45 ND). Die am häufigsten isolierten Bakterienfamilien waren *Pseudomonadaceae* (n=123/395; 31 %), *Enterobacteriaceae* (n=59/395; 15 %) und *Comamonadaceae* (n=54/395; 14 %). Es war offensichtlich, dass *Pseudomonadaceae* in der Mehrzahl der Isolate vor (n=88) und nach der Desinfektion (n=35) resultierte, gefolgt von *Stenotrophomonas* (n=16 VD, n=8 ND), Bacillus (n =19 VD und n=12 ND), *Comamonas* (n=15 VD und n=5 ND), *Citrobacter* (n=13 VD und n=7 ND) und *Acinetobacter* (n=8 jeweils VD und ND). Die relevantesten Bakterienarten, die vor und nach der Desinfektion isoliert wurden, waren Pseudomonas aeruginosa, die zur Risikogruppe 2 gehören, und *Comamonas testosteroni, Variovorax boronicumulans, Bacillus subtilis*-Gruppe und *Bacillus bataviensis*, die alle der Risikogruppe 1 zugeordnet sind. Zusammenfassend lässt sich sagen, dass die Wasserdesinfektion tatsächlich die Bakterienbelastung reduziert hat, aber nicht in allen Mastbetrieben, und es war keine deutliche logarithmische Verringerung der Bakterienzahlen in allen Einzelbetrieben erkennbar, die nach der Desinfektion der Tränkelinien beobachtet wurden.

9. TABLES AND FIGURES

10.APPENDIX

Table 11: Used materials and devices

Devices	
Bunsen burner	Schuett-biotec GmbH, Göttingen, Germany
Centrifuge 5424	Eppendorf AG, Hamburg, Germany
E count colony counter	Heathrow Scientific LLC., Illinois, USA
Freezer -20°C	Liebherr-International AG, Bulle, Switzerland
Freezer -80°C	Sanyo Electric Co., Ltd., Osaka, Japan
Gel Doc	Bio-Rad Laboratories Inc., Hercules, California
Heating block	Kleinfeld Labortechnik GmbH., Gehrden, Germany
Incubator 30°C	Ehret GmbH, Mahlberg, Germany
Incubator 37°C	3 M, Minnesota, USA
Incubator 42°C	Sanyo Electric Co., Ltd., Osaka, Japan
Merck Millipore Milli-Q™ Reference Ultrapure Water Purification System	Merck Millipore, Merck KGaA, Darmstadt, Germany
Microwave	Silva Schneider GmbH, Salzburg, Salzburg
Pipetus	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany
Power pac 1000 (gel electrophoresis)	Bio-Rad Laboratories Inc., Hercules, California
Scale	Tanita Corporation, Tokyo, Japan
Scale (for gel electrophoresis)	Santorius AG, Göttingen, Germany
Thermal cycler T100	Bio-Rad Laboratories Inc., Hercules, California
Consumables	
Agarose	VWR International GmbH, Darmstadt, Germany
Chelex Resin®	Bio-Rad Laboratories Inc., Hercules, California
Cryogenic vials 2ml	Biologix Group Ltd., Shandong, China
Deoxynucleoside triphosphate (dnTP) mix	Thermo Fisher Scientific Inc., Massachusetts, USA
Diethyl pyrocarbonate (DEPC)-treated water	Sigma-Aldrich Co., St. Louis, USA
Finnipipettes 1-1000µl	Thermo Fisher Scientific Inc., Massachusetts, USA
Glas pasteur pipettes	Brand GmbH & Co. KG, Wertheim, Germany
Inoculation loops 1,10µl	Sarstedt AG & Co. KG, Nürnbrecht, Germany
Latex gloves	Semperit Technische Produkte GmbH, Wien, Austria
Maxymum recovery tubes	Axygen Inc., California, USA
Nitrile gloves	Paul Hartmann GmbH, Wiener Neudorf, Österreich
PCR tubes (0.2ml)	NeoLab Migge GmbH, Heidelberg, Germany
PEQ Green	VWR International GmbH, Darmstadt, Germany
Platinum taq polymerase	Thermo Fisher Scientific Inc., Massachusetts,

	USA
Primer	Microsynth AG, Balgach, Switzerland
Safe-lock tubes 1,5ml, 2ml, 5ml	Eppendorf AG, Hamburg, Germany
Serological pipette 10ml, 25ml	Sarstedt AG & Co. KG, Nürnbrecht, Germany
Sterile filter tips 10µl, 100µl, 1250µl	Greiner-Bio One, Kremsmünster, Austria
Thermo Scientific™ GeneRuler™ 1kb DNA-ladder	Thermo Fisher Scientific Inc., Massachusetts, USA
Trisaminomethane (Tris) HCL	Merck KGaA,Darmstadt, Germany
Trisaminomethane-acetate-Ethylenediaminetetraacetic acid (TAE) buffer	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Culture media	
Violet-Red-Bile-Dextrose-Agar (VRBD)	Merck KGaA,Darmstadt, Germany
Trypto-Casein-Soy-Agar (TSA)	Biokar Diagnostics, Pantin Cedex, France
Charcoal-Cefoperozone-Deoxycholate-Agar (CCDA)	Thermo Fisher Scientific Inc., Oxoid Ltd., Massachusetts, USA
Xylose-Lysine-Desoxycholate-Agar (XLD)	Thermo Fisher Scientific Inc., Oxoid Ltd., Massachusetts, USA
Buffered Peptone Broth (BPW)	Biokar Diagnostics, Pantin Cedex, France
Brain Heart Broth (BHI)	Biokar Diagnostics, Pantin Cedex, France
Glycerol	Merck KGaA,Darmstadt, Germany
Bolton Broth (BB)	Thermo Fisher Scientific Inc., Oxoid Ltd., Massachusetts, USA
Thermo Scientific™ horse blood laked	Thermo Fisher Scientific Inc., Oxoid Ltd., Massachusetts, USA
Rappaport-Vassiliadis Soy Peptone Broth (RVS)	Thermo Fisher Scientific Inc., Oxoid Ltd., Massachusetts, USA
Bouillon Muller-Kaufmann tetrathionat novobiocin (MKTTn)	Biokar Diagnostics, Pantin Cedex, France