

Article

In Vitro Trial to Assess the Impact of Different Water Quality Parameters on the Stability of a Live Bivalent *Salmonella* Vaccine (*Salmonella* Enteritidis and *Salmonella* Typhimurium)

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Abstract

Zoonotic *Salmonella* strains are major pathogens causing foodborne illness, and poultry products are among the main sources of infection. Biosecurity on poultry farms is central to preventing the introduction of *Salmonella*; together with vaccination, it can reduce the risk of meat and eggs becoming contaminated. Live *Salmonella* vaccines are conveniently administered via drinking water, and good vaccination practices are essential to ensure flock protection. This requires drinking water to be compatible with live vaccines. Data about the impact of water quality on vaccine viability are limited; therefore, this study investigated the impact of different water parameters on the stability of a bivalent live *Salmonella* vaccine. In addition, the impact of the most common disinfectants used for drinking water sanitization was assessed. Fluoride, nitrate, sulphate levels, and hardness had little impact on vaccine survival, while aluminium, arsenic, iron, and manganese levels had a pronounced, dose-dependent effect. pH value, conductivity, and chloride levels impacted the vaccine stability only at high levels. Free chlorine, chlorine dioxide, and hydrogen peroxide strongly reduced the viability of the vaccine, even though a water stabilizer offered protection from chlorine and chlorine dioxide but not from hydrogen peroxide. These findings highlight the importance of drinking water quality for effective vaccination.

Keywords: *Salmonella*; live vaccine; drinking water; poultry



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1. Introduction

Salmonellosis remains one of the most important foodborne pathogens globally, causing a significant number of human illness cases each year and placing a substantial economic burden on the entire poultry industry. In 2023, the European Union reported 77,486 culture-confirmed human cases of *Salmonella* infection, while the United States documented 46,623 confirmed infections in 2016 [1,2], although the actual case number will be much higher due to under-reporting.

As in previous years, the three most commonly reported *Salmonella* serovars acquired in the EU in 2023 were *S. Enteritidis* (70.8%), *S. Typhimurium* (8.9%), and monophasic *S. Typhimurium* (5.1% of all human isolates), together representing almost 85% of all confirmed human cases [2]. Alarming, the World Health Organization (WHO) has

estimated that *Salmonella* may be associated with approximately 59,000 deaths worldwide each year and that it causes more deaths than any other zoonotic pathogen worldwide [3].

Among the various serovars of *Salmonella*, *S. Enteritidis* (SE) has consistently been the most prevalent, particularly in poultry and poultry products [2,4,5]. Specifically, eggs have been identified as a common source of infection for consumers. Starting in the 1980s, SE (in particular, phage type 4) emerged in European breeding and laying hen flocks, rapidly becoming the dominant serovar in poultry and the most common strain causing human infections. Similar situations occurred in the United States, albeit with different phage types [6–8]. In response, EU legislation now mandates that eggs from SE-positive hens cannot be sold as fresh table eggs and must undergo heat treatment. Consequently, an SE infection in a laying hen flock will result in substantial financial losses for the producer, often leading to early depopulation of the flock and the implementation of rigorous and costly cleaning and disinfection procedures [9]. Furthermore, recalls of affected poultry products contribute to significant economic losses and damage to the producer's reputation.

The implementation of various biosecurity measures to control infection in breeding and laying hens, including enhanced cleaning and disinfection procedures and more efficient rodent control, as well as the use of vaccines, has led to a notable reduction in human cases throughout Europe. These measures were initially introduced in selected European Union member states in the 1990s [10] before EU-wide legislation on *Salmonella* control in chickens and turkeys was finally implemented in 2003 [11]. *Salmonella* vaccination has proven particularly important to reduce transmission of *Salmonella* from poultry to humans via contaminated eggs or meat, therefore ensuring the safety of chicken-derived food products and minimizing economic losses for the producers. Besides good biosecurity and effective pest control, vaccination remains the most effective method for controlling *Salmonella* contamination in chickens at farm level [12]. In almost all European member states, as well as in the UK, vaccination of breeding flocks and laying hens is commonly practiced, and in some countries, such as Germany, Austria, and Belgium, all laying hens are required by law to be vaccinated against SE. Vaccination has undeniably been an effective measure in reducing the risk of *Salmonella* infection, leading to a relevant decrease in SE prevalence both in chicken flocks and in the human population [7,8,13,14]. Oral administration of ST vaccines at day of hatching has also been shown to control *Salmonella* infections in broilers [15]. In other parts of the world, notably Asia and Africa, vaccination of laying hens is so far only rarely used, and vaccination focuses mostly on broiler breeder flocks. The reason for this difference in vaccination adoption is, in most cases, a lower level of food safety awareness in developing and/or lower-income countries, even though the burden of illness caused by poultry-related *Salmonella* is high in every region [3].

Both inactivated and live attenuated *Salmonella* vaccines are commercially available and used in modern poultry farming. While inactivated vaccines primarily stimulate humoral immunity through the production of IgM and IgY in the bloodstream, live attenuated *Salmonella* vaccines trigger cellular and humoral immune responses mostly in the gut, providing enhanced protection for poultry [16,17]. The practice of administering live poultry vaccines through drinking water has a well-established history and is a widely adopted vaccination approach [18]. This technique offers numerous benefits. As well as being a relatively uncomplicated and speedy method for providing the vaccine to a considerable number of birds, it also ensures minimal stress for the chickens and proves economically advantageous for the farmer because of low labour costs. However, despite its apparent simplicity, this method demands attention to detail and well-trained staff to achieve desired outcomes.

In fact, the most common cause of vaccine failure in poultry is poor administration practice [19]. If a live vaccine is not administered *lege artis*, the homogenous dispersal of

the vaccine and, as a result, the consistency of the immune response of individual birds may be jeopardized. Therefore, adherence to best practices for vaccination, including diligent preparation and administration of the vaccine, is essential for effective immunization. However, it is also possible that vaccination failures are the result of the presence of noxious substances in the drinking water or the drinking water line, potentially threatening the vaccination success of a whole flock. Since the German Guideline on Oral Use of Veterinary Medicinal Products in the Livestock Sector via Feed or Water was implemented in May 2014, the need for practical management of drinking water has been increasing [20]. It is important that water pipes are dimensionally designed and installed to prevent biofilm formation by providing a sufficient flow rate and properly located pipes. Recommendations on drinking water quality have also been adopted at the European level. In August 2020, the European Medicines Agency (EMA) published a scientific problem analysis and some recommendations to ensure the safe and the efficient administration of oral veterinary medicinal products via routes other than medicated feed [21]. It states that the management of water quality and knowledge of its physio-chemical and microbiological properties are important for correct medication. Therefore, it is crucial to understand the key requirements for the quality of drinking water used to distribute vaccines, including the impact of physio-chemical parameters and the residues of chemicals or disinfectants.

A basic understanding of the importance of good water quality, both for the benefit of animal health and the successful application of substances, is already present among the farming community, but in some cases, detailed knowledge about the interaction between the most important parameters in drinking water and live vaccines is still lacking.

The aim of this study was therefore to investigate the influence of different water quality parameters and some selected disinfectants used for water sanitization on the viability of a live bivalent *Salmonella* vaccine (AviPro™ SALMONELLA DUO) for poultry. Several potential drinking water contaminants, as well as disinfectants and their possible interference with the viability of the vaccine, were tested in vitro. The results will prove beneficial for anyone using live drinking water vaccination in poultry production.

2. Materials and Methods

A series of in vitro trials was conducted to assess the effect of physio-chemical and chemical (dissolved ions and metal) parameters, as well as common disinfectants, on the stability of a live *Salmonella* vaccine over a 24 h period. The data were generated from May 2024 to February 2025.

2.1. Vaccine

The live attenuated bivalent vaccine AviPro™ SALMONELLA DUO (Elanco Deutschland GmbH, Bad Homburg, Germany; Batch number: F0799D; F1044E, F0991D) was used throughout the trial. The vaccine contains at least 1×10^8 colony-forming units (CFUs) of a live attenuated *Salmonella* Enteritidis strain (SE, strain Sm24/Rif12/Ssq) and at least 1×10^8 CFU of a live attenuated *Salmonella* Typhimurium strain (ST, strain Nal2/Rif9/Rtt) per dose. In addition, the vaccine contains additives such as soy peptone, sucrose, gelatine, and HEPES buffer.

2.2. Detection of *Salmonella* Vaccine Strains from the Water Samples

The two vaccine strains can be grown on selective and non-selective media, and they can be differentiated from other organisms and from *Salmonella* field strains by using a defined pattern of sensitivities/resistances to certain antibiotics. Granucult Plus Standard 1 Agar (S1A, Merck KGaA, Gillingham, UK, product code 1.03864.0500) was used as a nonselective medium, as only a negligible number of bacteria are expected to be present in

tap water, and it was therefore not necessary to use selective plates (such as Brilliant Green Agar or BPLS agar). To differentiate between the two vaccine strains, one set of plates containing 100 µg/mL streptomycin (Sigma UK, Hampshire, UK, product code S9137) was used to support the growth of the SE vaccine strain and to exclude the ST vaccine strain, and another set of plates containing 5 µg/mL nalidixic acid (Sigma UK, Hampshire, UK, product code N8878-5G) was prepared to support the growth of the ST vaccine strain and to exclude the SE strain.

2.3. Water Quality Parameters

2.3.1. Physio-Chemical Parameters

The pH of the tap water used was checked using a Hanna HI 8314 pH meter (Hach UK, Manchester, UK), which had been calibrated at pH 4.01 and 7.01 before use. Using 0.1 Mol/L (0.1 N) HCL (Merck UK, Product code 1.09060.1000) and 0.1 Mol/L (0.1 N) NaOH (Merck, Product code 1.09141.1003), 6 × 1 L of tap water had their pH values adjusted to produce stock solutions of pH 4, 5, 6, 7, 8, and 9 (Table 1).

Table 1. Physio-chemical parameters tested with the AviPro™ SALMONELLA DUO vaccine.

Chemical	Value	Level					
Temperature	°C	15	30				
pH Value		4	5	6	7	8	9
Conductivity	µS/cm	30	300	3000			
Hardness	mg/L CaCO ₃	50	100	200	400		

The Merck Supelco 3000 µS Conductivity Standard Solution was diluted with deionised lab-grade Type 2 water to produce the required conductivity concentrations of 30, 300, and 3000 µS/cm (Table 1). Concentration levels were confirmed using a Hanna Conductivity Meter Model HI 98129 and Hanna Conductivity Calibration buffer HI7031L 1413 µS/cm.

The Merck Supelco Standard Hardness solution 1000 mg/L was diluted with deionised lab-grade Type 2 water to produce the following solutions: 50 mg/L CaCO₃, 100 mg/L CaCO₃, 200 mg/L CaCO₃, and 400 mg/L CaCO₃ (Table 1). Each solution was checked using a Hanna HI-83300-02 Multiparameter photometer, using Hanna hardness testing kits HI-93720-01 and HI93735-00.

2.3.2. Chemical Parameters

The following chemicals standards were diluted in deionised lab-grade Type 2 water to produce the concentrations as described in Table 2: Aluminium Merck Supelco ALUMINIUM Standard Solution 1000 mg/L—Product Code 1.19770.0100 Lot HC32535870; Arsenic Merck Supelco ARSENIC Standard Solution 1000 mg/L—Product Code 1.19773.0500; Chloride Merck Supelco CHLORIDE Standard Solution 10,000 mg/L—Product Code 102747213; Fluoride Merck Supelco FLUORIDE Standard Solution 1000 mg/L—Product Code 77365-100ml; Iron Merck Supelco IRON Standard Solution 1000 mg/L—Product code 1.19781.0100; Manganese Merck Supelco MANGANESE Standard Solution 1000 mg/L—Product Code 1.19789.0100; Nitrate Merck Supelco NITRATE Standard Solution 1000 mg/L—Product Code 1.19811.0500; Sulphate Merck Supelco SULPHATE Standard Solution 1000 mg/L—Product Code 1.19813.0500.

Table 2. Chemical parameters tested with the AviPro™ SALMONELLA DUO vaccine.

Chemical	Value	Level			
Aluminium	ppm	0.2	0.5	3	6
Arsenic	ppm	0.01	0.02	0.05	
Chloride	ppm	100	250	500	1000
Fluoride	ppm	1	2	4	
Iron	ppm	0.5	1	5	10
Manganese	ppm	1	2	4	8
Nitrate	ppm	100	200	300	
Sulphate	ppm	100	500	1000	

2.3.3. Chemical Water Disinfectants

Free Chlorine

A sodium hypochlorite solution was diluted with deionised sterile lab-grade Type 2 water to produce 500 mL of each of the standard concentrations to be used. The free chlorine levels tested are shown in Table 3.

Table 3. Free chlorine levels tested with the bivalent AviPro™ SALMONELLA DUO vaccine.

Chemical	Value	Level								
Chlorine	ppm	0.2	0.4	0.6	0.8	1.0	1.5	2.0	5.0	10.0

Chlorine Dioxide

For this trial, water samples were collected from a broiler farm where the mains-fed water supply had undergone chlorine dioxide treatment using a commercial chlorine dioxide generator (Integrated Water Services, Green Lane, Walsall, UK).

Water samples were collected and transported to the laboratory for testing without any delay and testing commenced within 30 min of chlorine dioxide production to ensure that no degradation of the chlorine dioxide produced occurred. Sampling containers were filled right to the top, with no airspace left, to inhibit any gaseous exchange.

The water stabilizer AviBlue™ was added to half of the samples, and all samples were duplicated to enable testing at 15 and 30 °C.

The addition of vaccine and the titration and analysis of each treatment over a four-hour time period were the same as the method carried out for the chemical parameters assayed.

Hydrogen peroxide

A commercially available hydrogen peroxide water treatment product (Watersan® Silver Stabilized Hydrogen Peroxide, RS Hygiene Ltd., Worlington, UK) was used at the manufacturer's recommended rate for treating chicken drinking water lines with birds present in the house (1:20,000).

The addition of vaccine and the titration and analysis of each treatment over a four-hour time period were the same as the method carried out for the chemical treatments assayed, with the exception that the vaccine dosage rate used was modified to reflect the proposed dosage for 50-week-old laying chickens. All samples were duplicated to enable testing at 15 and 30 °C.

2.3.4. Stabilizer Used with All Parameters

The water stabilizer AviBlue™ (Elanco Deutschland GmbH, Bad Homburg, Germany, Batch No. 140719, 143544) was used according to the manufacturer's recommendations (125 g AviBlue™ per 1000 L water).

2.3.5. Experimental Procedure

For all parameters tested, the test procedure was the same and is described in the following paragraph.

For each parameter to be tested, 4 × 250 mL beakers with lids were prepared by adding 100 mL of the parameter being tested.

AviBlue™ was added to two labelled beakers at the manufacturer's in-use concentration. Then, one pot containing tap water and one pot containing tap water plus AviBlue™ were put in a 30 °C incubator to equilibrate their temperatures; the other two were kept at 15 °C in a cooled incubator, resulting in the following trial samples:

- 15 °C Tap Water;
- 15 °C Tap Water + AviBlue™;
- 30 °C Tap Water;
- 30 °C Tap Water + AviBlue™.

A vial of the vaccine was prepared by adding 9 mL of tap water to the 2000-dose vial, producing a suspension with a concentration of 200 doses per 1.0 mL. A total of 10 doses of this vaccine solution were added to each test beaker, producing a vaccine concentration of 1 dose per 10 mL, mimicking the recommended rate for day-old chicks.

A control was set up using normal tap water plus vaccine, as above, and kept at room temperature (18–22 °C) for the duration of the trial.

Each test beaker was then assayed using serial dilutions in Maximum Recovery Diluent (SGL), over the 10^{-1} to 10^{-6} range.

Each dilution was then plated out by taking 100 µL from each dilution and plating onto each agar plate to allow for the enumeration of both SE and ST separately, resulting in a test range of 10^{-1} to 10^{-7} .

After incubation at $37\text{ °C} \pm 1\text{ °C}$ for between 24 and 48 h, colonies were counted on the plates, which showed between 30 and 300 colonies.

Each recorded count was calculated as the average from duplicate plates to provide a final count of CFU/mL.

Each pot was kept at its designated temperature and then retested at 4, 8, 12, 16, and 24 h after preparation (for the stability trial) and at 2 and 4 h, respectively (for all other trials).

3. Results

3.1. Impact of Distilled Water and Tap Water on Bivalent Vaccine

Stability Throughout a 24 h Period

The aim of this investigation was to monitor levels of the AviPro™ SALMONELLA DUO vaccine in water over a 24 h period, mimicking conditions in a drinking water line for day-old chicks, and to assess any potential deterioration. When comparing distilled water and tap water, no pronounced differences in vaccine stability were observed. Both the SE and ST fractions demonstrated stability throughout the 24 h trial period, and the addition of AviBlue™ did not provide any measurable benefit under these conditions. Vaccine counts were still within the acceptable range according to the Summary of Product Characteristics at both temperatures (15 and 30 °C) after 24 h, although a slight decline was seen at the 16 h and 24 h time points. The results at 15 °C are shown in Figure 1.

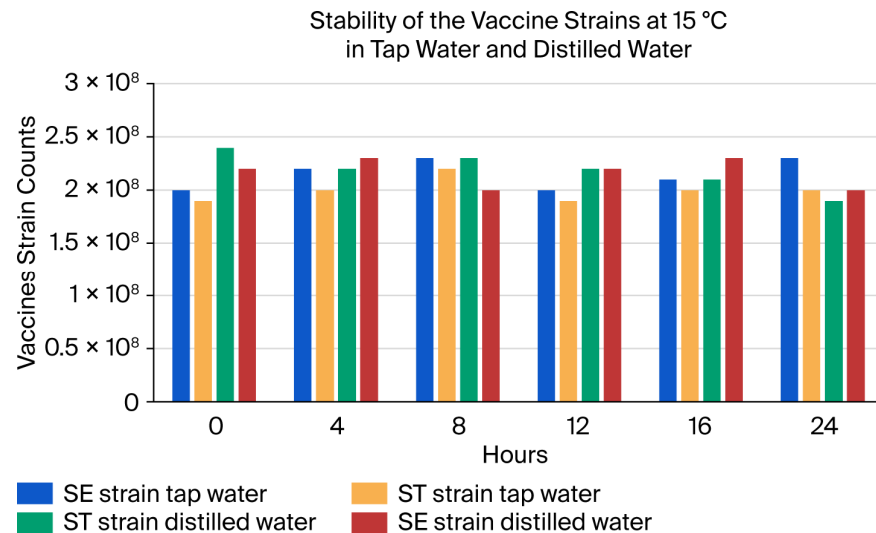


Figure 1. Stability of AviPro™ SALMONELLA DUO in distilled and tap water over a 24 h period.

3.2. Impact of Physio-Chemical Parameters on Bivalent Vaccine

3.2.1. Impact of pH Value

The results demonstrate acceptable stability levels of AviPro™ SALMONELLA DUO between pH 5 and pH 8. A decline in vaccine counts over time was observed at both ends of the pH range investigated, specifically at pH 4 and 9. While there is some evidence to suggest that AviBlue™ may be able to mitigate some of these reductions, the protective effect seems to be minimal.

3.2.2. Impact of Conductivity

The results show the stability of AviPro™ SALMONELLA DUO at varying conductivity levels. A slight reduction in vaccine levels was observed after 24 h at a conductivity of 3000 µS/cm, with a slightly lower effect in samples containing AviBlue™; differences, however, were small. Vaccine levels for both strains at all conductivity levels (30 µS/c, 300 µS/c, 3000 µS/c) were still within the manufacturer's dose requirements.

3.2.3. Impact of Hardness

The stability of AviPro™ SALMONELLA DUO in the presence of varying Standard Hardness solutions was observed. The hardness ranges chosen (50 mg/L CaCO₃, 100 mg/L CaCO₃, 200 mg/L CaCO₃, 400 mg/L CaCO₃) appear to have little or no effect on vaccine counts over a 24 h period. It can generally be assumed that the vaccine remains stable under in vitro conditions across variable water hardness values and that successful vaccination can be achieved within the water hardness range typically observed in drinking water.

3.3. Impact of Dissolved Ions on Bivalent Vaccine

3.3.1. Impact of Chloride

Neither the SE nor the ST fraction was impacted at chloride concentrations of 100 and 250 mg/L. At 500 mg/L, a minor reduction over time was observed for both fractions, with some protective effect from AviBlue™ in the standard solution. At the highest concentration tested (1000 mg/L), the impact of chloride was more pronounced towards the end of the trial period, and AviBlue™ provided correspondingly greater protection. However, these effects were minimal overall, and vaccine levels for both SE and ST fractions remained within the manufacturer-specified range throughout the study. These results indicate that the AviPro™ SALMONELLA DUO vaccine is largely stable across a wide

range of chloride concentrations, with AviBlue™ offering only minor additional protection at the highest levels.

3.3.2. Impact of Fluoride

Both vaccine strains were observed to be stable across all three fluoride levels tested (1 mg/L, 2 mg/L, 4 mg/L), with no pronounced differences between solutions with and without the addition of AviBlue™.

3.3.3. Impact of Nitrate

Throughout the trial, the different nitrate concentrations (100 mg/L, 200 mg/L, 300 mg/L) appeared to have little to no effect on the vaccine, with both the SE and ST fractions remaining largely stable over the full 24 h period. These observations suggest that the AviPro™ SALMONELLA DUO vaccine is highly resilient to the range of nitrate levels tested, with no pronounced deterioration observed under the conditions chosen in this study.

3.3.4. Impact of Sulphate

As to the different sulphate levels (100 mg/L, 500 mg/L, 1000 mg/L), very little effect on either the SE or the ST fraction of the vaccine was observed. A slight reduction at the 24 h test point was seen, but the levels for both SE and ST were still within the range of the manufacturer-specified vaccine criteria for AviPro™ SALMONELLA DUO. Consequently, with little or no effect, the addition of AviBlue™ did not impact vaccine recovery.

3.4. Impact of Dissolved Metals on Bivalent Vaccine

The impacts of the dissolved metals tested on the vaccine are shown in Table 4. Light grey fields indicate a marked negative effect (a reduction of 50% or more) on the vaccine recovery of at least one of the two vaccine strains. Dark grey fields indicate that less than 10% of viable vaccine organisms (of at least one of the two strains) could be recovered. The asterisk indicates that the addition of the water stabilizer could mitigate the effect at the given concentration/time point.

3.4.1. Impact of Aluminium

The stability of the SE and ST vaccine fractions was evaluated over 24 h at 15 °C and 30 °C in the presence of varying aluminum concentrations (0.2 mg/L; 0.5 mg/L; 3 mg/L; 6 mg/L), with and without the stabilizer AviBlue™ (Table 4). At 0.2 mg/L, which is in accordance with German drinking water legislation [22], SE showed a slight decrease at 15 °C, while ST remained stable, and both fractions experienced minor reductions at 30 °C. At a concentration of 0.5 mg/L, the vaccine viability was markedly impacted, with a more severe effect at the higher temperature, but the addition of AviBlue™ protected both vaccine strains. Above a concentration of 0.5 mg/L, vaccine viability was severely reduced at both temperatures, and the addition of the water stabilizer did not show a protective effect. These findings highlight the critical importance of controlling aluminum levels to ensure vaccine efficacy.

3.4.2. Impact of Arsenic

The stability of the SE and ST vaccine fractions was evaluated over 24 h at 15 °C and 30 °C in the presence of varying arsenic concentrations (10 µg/L, 20 µg/L, 50 µg/L), with and without the stabilizer AviBlue™ (Table 4). Arsenic had a moderate effect on both the SE and the ST fractions of the AviPro™ SALMONELLA DUO vaccine, with the effect increasing with concentration. The lowest concentration tested (10 µg/L), which is the upper limit for drinking water for human consumption in Germany [22], led to a

slight reduction in vaccine strain counts, and the effect could be mitigated through the addition of a water stabilizer. At a concentration of 20 µg/L, the effect was slightly more pronounced but could still be compensated through the addition of a water stabilizer. Only at a concentration of 50 µg/L could the water stabilizer no longer mitigate the effect after 12 h.

3.4.3. Impact of Iron

The stability of the SE and ST vaccine fractions was evaluated over 24 h at 15 °C and 30 °C in the presence of varying iron concentrations (0.5 mg/L, 1 mg/L, 5 mg/L, 10 mg/L), with and without the stabilizer AviBlue™ (Table 4). High iron concentrations had a severe impact on vaccine recovery, with viable vaccine counts being reduced to below 10% of the starting count from 5 mg/L onwards, but a negative effect was already seen at lower concentrations in a time- and temperature-dependent manner. After 4 h, vaccine counts were still satisfactory at an iron concentration of 1 mg/L, but from 8 h onwards, the effect of iron was detrimental and could hardly be mitigated through the addition of a water stabilizer. These results show that iron concentration in drinking water needs to be carefully monitored and that vaccination might not be successful if iron levels reach 1 mg/L and if vaccination takes longer than 4 h to be completed.

3.4.4. Impact of Manganese

The stability of the SE and ST vaccine fractions was evaluated over 24 h at 15 °C and 30 °C in the presence of varying manganese concentrations (1 mg/L, 2 mg/L, 4 mg/L, 8 mg/L), with and without the stabilizer AviBlue™ (Table 4). At 1 mg/L, a slight reduction was observed at 15 °C for both fractions, and AviBlue™ could mitigate the effect for a period of 16 h. At 30 °C, a pronounced reduction occurred after 8 h, and AviBlue™ could protect the vaccine until 12 h. At 2 mg/L, vaccine counts were only satisfactory at 15 °C and for 4 h, while the higher temperature already had a detrimental effect after 4 h. From 4 mg/L onwards, vaccine counts were reduced to less than 10% after 4 h regardless of water temperature. Interestingly, the addition of the water stabilizer had very little protective effect in the presence of high manganese levels.

Table 4. Impact of dissolved metals on bivalent *Salmonella* vaccine.

Parameter	Concentration	Temperature	Time (h)				
			4	8	12	16	24
Aluminum	0.2 mg/L	15 °C				*	*
		30 °C			*	*	*
	0.5 mg/L	15 °C				*	*
		30 °C		*	*	*	*
	3 mg/L	15 °C					
		30 °C					
	6 mg/L	15 °C					
		30 °C					
Arsenic	10 µg/L	15 °C				*	*
		30 °C					*
	20 µg/L	15 °C					*
		30 °C			*	*	*
	50 µg/L	15 °C		*			
		30 °C	*	*	*		

Table 4. Cont.

Parameter	Concentration	Temperature	Time (h)				
			4	8	12	16	24
Iron	0.5 mg/L	15 °C					*
		30 °C			*		
	1.0 mg/L	15 °C		*	*		
		30 °C					
	5.0 mg/L	15 °C					
		30 °C					
	10.0 mg/L	15 °C					
		30 °C					
Manganese	1 mg/L	15 °C			*	*	
		30 °C		*	*		
	2 mg/L	15 °C					
		30 °C	*				
	4 mg/L	15 °C					
		30 °C					
	8 mg/L	15 °C					
		30 °C					

* Benefit from water stabilizer. Light grey fields indicate a pronounced negative effect (−50%) on the vaccine recovery of at least one of the two strains. Dark grey fields indicate that less than 10% of the viable vaccine organisms of at least one of the two strains could be recovered.

3.5. Impact of Water Disinfectants on Bivalent Vaccine

3.5.1. Impact of Free Chlorine

Up to 0.6 mg/L free chlorine, our trials showed no major impact on either of the vaccine strains.

From 0.8 mg/L onwards, depending on temperature, vaccine levels started to drop off, and at 2 mg/L of free chlorine, a dramatic reduction in both the SE and the ST strain levels was observed at 15 °C, with an even steeper reduction seen at 30 °C. At 30 °C, a marked effect on both strains was already observed at time point 0, and no culturable vaccine could be detected after 4 h.

However, the solution containing AviBlue™ showed superb vaccine protection with stable results over the 24 h period for both vaccine strains. Free chlorine levels of 5 mg/L and 10 mg/L resulted in virtually no culturable vaccine, even at time 0, yet the solution with AviBlue™ again shows vaccine protection, with stable vaccine counts recovered. These results show that free chlorine has a detrimental effect on vaccine viability even at low levels, but that the use of a water stabilizer can offer satisfactory protection even at levels which would normally only be seen during spike periods. The results suggest that the end point for free chlorine in water without a stabilizer, such as AviBlue™, is somewhere between 1.0 and 1.5 mg/L, and that a substantial reduction in vaccine counts should be expected if free chlorine levels reach 1 mg/L or higher.

3.5.2. Impact of Chlorine Dioxide

Water that had undergone chlorine dioxide treatment contained less than 1000 cfu of each of the vaccine strains per mL, indicating that the treatment had a detrimental effect on the vaccine strains. However, after the addition of AviBlue™, no marked drop in vaccine levels was observed. It is therefore important for farmers to know that chlorine dioxide

treatment needs to be stopped before vaccination, but that the addition of a water stabilizer can mitigate the negative effects if needed.

3.5.3. Impact of Hydrogen Peroxide

Hydrogen peroxide, when applied at the manufacturer's recommended rate for treating chicken drinking water lines with birds present in the house (1:20,000), led to a detrimental effect on the AviPro™ SALMONELLA DUO vaccine, affecting both the SE and ST fractions equally. At a water temperature of 15 °C, a reduction of more than 99% of vaccine counts was observed for both strains after 2 h, and this negative effect was further exacerbated at the elevated temperature of 30 °C. The addition of AviBlue™ did not provide any measurable protective effect against the deleterious action of hydrogen peroxide. The hydrogen peroxide treatment of the drinking water therefore needs to be stopped well in time before vaccination.

4. Discussion

Commercial poultry production has a high risk of encountering infectious diseases, especially under intensive farming conditions. In order to mitigate that risk, biosecurity has become a key component of modern poultry production, aiming to prevent the introduction and spread of infectious agents that threaten animal health, as well as those agents that are relevant for food safety. *Salmonella* spp. are among the pathogens of major concern in the context of food safety due to their zoonotic potential and their ability to persist for several months in poultry populations and farm environments [10,23]. In a previous study, primary biosecurity was defined as the prevention of pathogen transmission between farms, secondary biosecurity as the prevention of pathogen spread within a farm, and tertiary biosecurity as measures that enhance animals' resistance or immunity against pathogens, such as vaccination [24]. The implementation of effective vaccination strategies against *Salmonella* is therefore an essential component of the comprehensive biosecurity management of poultry farms.

Salmonella vaccine programs rely on injectable inactivated vaccines and on live attenuated vaccines administered via water [25]. Generally, live vaccines are considered more effective in boosting cellular immunity [17,26]. Some live *Salmonella* vaccines even offer a dual mode of protection by enhancing mucosal immunity and by inducing a colonization inhibition effect against wildtype *Salmonella* strains [15,27]. These two mechanisms work together to significantly reduce colonization and excretion, which can only be achieved by applying the first vaccine dose as early as possible, preferably on the first day of life [17]. The administration of vaccines via drinking water has also become increasingly popular in poultry production for several other reasons. First of all, the oral application route ensures a stress-free application without the need to catch and inject birds and there are also no complications from tissue damage due to injection. Oral application leads to no depression or dip in production parameters, as is expected for most injectable vaccines. Furthermore, oral application of *Salmonella* vaccines follows the natural route of infection rather than an artificial parenteral route and therefore initiates an immune response at the natural site of infection, i.e., the gut.

When using live vaccines applied via drinking water, it is important to educate users of such vaccines that they need to pay special attention to the quality of the drinking water, as certain parameters may have a detrimental effect on the viability of the vaccines. It has been observed that poor water quality can potentially reduce the effectiveness of vaccines and medication administered through water lines [28]. Monitoring the viability of live vaccines is already practiced by many users and is a useful tool with which to prove the successful application of a vaccine, but previous studies have focused on the efficacy of

vaccines in poultry, rather than investigating the potential impact of water components on the viability of the vaccine. Therefore, it was of interest to investigate the effect of various water parameters on a live *Salmonella* vaccine administered via drinking water.

Water quality varies greatly between geographical regions, being dependent on the source of water and water treatment options. Drinking water sources for poultry can be mains (i.e., municipal) water, borehole water, water from natural or artificial ponds/lakes, or even water sourced from rivers and streams. Therefore, the microbiological and chemical properties of the water supply will vary greatly depending on the source and season (i.e., dry versus rainy season, cold versus hot season). Field experience has further demonstrated that variations in water quality, both between different farms and even within a single complex from one well to another, can lead to noticeable differences in bird performance, behaviour, and welfare [29].

Across the EU, the Drinking Water Directive (EU) 2020/2184 sets general standards, but national parameters vary, and no binding limits exist for drinking water for farm animals. Non-mandatory guidelines, such as those from the German Federal Ministry of Food, Agriculture and Consumer Protection (BMEL), based on scientific recommendations, serve as useful guidelines for farmers to assess the water quality on their farms [30]. However, guidance on how specific water parameters affect the suitability of water for intensively reared poultry, especially when used for vaccination, is limited. Ensuring that the basic hygiene and chemical quality of the water meet the birds' needs remains essential. The presence of multiple elements causing poor water quality highlights the importance of considering their interactions rather than just their individual presence [31]. In a previous study, it was found that the percentage of samples exceeding German chemical guideline values (64.1%) was higher compared to the percentage of samples exceeding German microbiological guideline values (47.6%) [32]. This suggests that there may be a greater need for monitoring and addressing chemical contaminations in the samples analyzed, as they were found to exceed the recommended guidelines more frequently than microbiological contaminants. An Italian study found that tap and well water largely met human drinking water standards, although both sources showed high iron and hardness levels [33]. The influence of chemical parameters on substances administered via drinking water, such as vaccines, is therefore highly relevant.

In the present study, the main impact on the bivalent *Salmonella* vaccine was seen in the presence of high levels of aluminium, arsenic, iron, and manganese. These levels had a pronounced, dose-dependent impact on the survival of the vaccine, even though it has to be noted that the study was performed *in vitro* and that the performance of the vaccine *in vivo* might differ from the *in vitro* results. Our observations are similar to those of studies demonstrating that metallic water can reduce the effectiveness of drugs (e.g., tetracyclines) or vaccines (e.g., Newcastle Disease) [34,35]. Several methods to remove metals from drinking water, such as aeration or ion exchange, may be used in cases where levels are high [36–39].

The water stabilizer AviBlue™ was able to mitigate the negative effect of aluminium and arsenic on the vaccine at lower concentrations, while it had very little protective effect in the presence of high iron or high manganese levels.

Globally, inorganic arsenic in groundwater-based drinking water is a major public health risk as it increases the risk of cancer, as well as other undesirable health effects [40–42]. Therefore, the World Health Organization (WHO) has lowered its guideline value accordingly, from 200 to 50 µg/L in 1963 and from 50 to 10 µg/L in 1993 [43]. The limit value for arsenic in drinking water in Germany and the UK is currently 10 micrograms per litre (µg/L) [22,44]. Since a negative effect on the viability of the vaccine was only observed at higher levels, it is safe when using tap water that is within the official limits.

However, in other regions, such as Bangladesh, the contamination of groundwater by arsenic is considered to be a significant health issue, and levels may exceed recommended limits [45].

Aluminum is present in all natural waters and waterworks, occurring in various organic and inorganic forms depending mainly on pH [46,47]. Natural waters typically contain 0.0001–1 mg/L aluminium, while acidic waters ($\text{pH} < 5$) may exceed 100 mg/L. Because solubility is lowest at pH 6–8, surface and groundwater usually show only 60–300 $\mu\text{g/L}$ [48]. Elevated aluminium levels in tap water often arise from improper coagulation. Since high concentrations may pose risks to human health, including potential links to Alzheimer-type brain changes [49], residual levels in drinking water should be kept minimal. Therefore, the Environmental Protection Agency (EPA) recommends a Secondary Maximum Contaminant Level of 0.05–0.2 mg/L in drinking water [50], while the WHO sets a limit below 0.2 mg/L [51]. Some countries apply stricter standards, e.g., ≤ 0.2 mg/L in Germany [22]; ≤ 0.1 mg/L in France, Canada, Japan, and Sweden [47]; and ≤ 0.05 mg/L in the United States [52]. Guideline values for aluminium in livestock drinking water generally range from 3.6 to 5.6 mg/L, with a common reference value of 5 mg/L [53]. Our results show that such high aluminium levels are not compatible with a live *Salmonella* vaccine if vaccination takes longer than 4 h to complete.

Iron is a common metal that can occur in higher concentrations in drinking water. Regulatory limits are 0.2 mg/L in Germany [22] and the UK [54] and 0.3 mg/L according to the WHO [51], albeit primarily for aesthetic and technical reasons such as taste, staining, and pipe deposition, rather than health risk. In the context of livestock, the German Federal Ministry of Food, Agriculture and Consumer Protection [20] advises a maximum iron concentration of 3 mg/L [20]; however, in our study, iron levels from 0.5 mg/L onwards already led to a decline in vaccine counts, meaning that the vaccine would be compromised at levels which are well within the range of the German guidelines. It has previously been shown that poultry can tolerate high levels of iron, with up to 600 mg/L not having a negative impact on performance [55]. While such high iron levels may be rare, iron levels in well water on poultry farms have been found to reach up to 115 mg/L [55]. In another study, iron levels in water samples ranged from 0.03 to 101 mg/L, with 32.8% exceeding the German federal guideline for livestock (3 mg/L) [32]. According to these data, the *Salmonella* vaccine would be affected by iron in one-third of the farms. However, most data were generated for swine farms, and only 36.6% of poultry farms used well water [32]. Indeed, a previous study mentioned that high iron levels have a negative impact on the vaccine; however, no threshold values were provided in this publication [56]. It is also known that higher levels of iron in drinking water can affect acceptance and thus reduce water intake in livestock [57]. In addition, iron has been reported to support the growth of *E. coli* and coliforms [58–60]. Based on the findings of this study, it is recommended that water treatment systems be installed to help remove chemicals from the drinking water, and several different techniques are in use to achieve this. These include ion exchange and water softening, the use of activated carbon and other filtration materials, supercritical fluid extraction, bioremediation, and limestone treatment [36,61–64]. Other methods include oxidation by aeration, chlorination, or ozonation, followed by filtration, or using ash, aerated granular filters, or adsorption [65–68]. Aeration and separation are the most commonly used methods for iron removal in public water systems [37]. However, de-ironing may not be applicable to all types of well water. Therefore, it is advisable to take a sample of the water first and to consult with the different suppliers available on the market.

Manganese is a common metallic element typically found in air, soil, food, and water. Even though it is a vital nutrient, excessive levels can disrupt the normal functioning of the

nervous system [69]. In occupational settings, long-term exposure to manganese has been identified as a potent neurotoxic substance, capable of causing motor and cognitive deficits and neuropsychiatric symptoms in humans [70]. More recent studies have investigated the health hazards associated with environmental exposure to this metal, suggesting potential psychological and neurological abnormalities [71]. Children are thought to be particularly vulnerable due to the ongoing development of their nervous system, a higher exposure dose relative to body weight, and their immature homeostatic mechanisms [72]. Therefore, the WHO recommends a health-based maximum guideline value of 0.4 mg/L manganese in drinking water [51], while the German Drinking Water Ordinance [22] sets a more stringent limit of 0.05 mg/L, reflecting additional esthetic and technical considerations. For animals, recommended thresholds are considerably higher at <4 mg/L [20]. This study demonstrated, however, that vaccine stability is highly sensitive to manganese concentration and that, depending on exposure time and temperature, even manganese levels of 1 mg/L can jeopardize vaccine efficacy, which would be well within the recommended range for farm animal drinking water. There are numerous areas in North America and other parts of the world where water contains high levels of manganese [73]. Groundwater can naturally contain high levels of manganese due to the erosion and seepage of manganese-containing minerals. Even though AviBlue™ can provide partial protection at low-to-moderate manganese levels, little to no benefit was observed at higher concentrations. In addition, similar to iron, manganese can cause deposits in the water line and interfere with the effectiveness of active ingredients. Consequently, water treatment systems may also be appropriate for manganese, even if the water is within the range of values suitable for livestock according to German guidelines.

Physio-chemical parameters such as pH value, temperature, conductivity, dissolved oxygen, and the concentration of dissolved substances (organic/inorganic) have a significant impact on the behaviour and effectiveness of active substances in water as they determine solubility, stability (hydrolysis, photolysis), adsorption (e.g., to sediments), and biological availability (bioavailability) [28,74,75]. For example, polar parameters such as high pH or salts increase the solubility of many active substances, while temperature and organic substances influence the degradation pathways [76].

The pH is a measure used to determine the amount of hydrogen ions present in a given medium, indicating whether it is acidic, neutral, or alkaline. While water pH has no direct impact on consumers, and no health-based standards have been set, it remains one of the most important indicators of water quality. Even though the precise effects of the pH on water intake or animal health are unknown, it is generally considered that water with a pH value of 6–9 is suitable for livestock consumption [30]. For laying hens, a pH of 6–6.8 is recommended [77]. This range proved favourable for the AviPro™ SALMONELLA DUO vaccine, which showed a marked decline in viability over time only at either extreme of the pH range (i.e., pH 4 and 9), and this is in line with a previous study where a pH of 9 had a pronounced impact on vaccine survival [56]. However, previous studies have also shown that low pH reduces performance and decreases eggshell quality, while also making the water unpalatable and corroding the metal components of the watering system [78,79]. It has also been shown that lower drinking water pH reduces the survival and growth of *Salmonella* in birds and water systems [80]. Conversely, alkaline water with a pH above 7.5 may damage equipment, while a pH above 8.5 could cause mineral incrustation [81]. Seasonal shifts in rainwater and reservoir water pH are documented, ranging from 5.7 in spring and 5.8 in summer to 4.6 in autumn and 4.5 in winter [82], which may affect vaccine administration via drinking water. This may need to be considered when applying vaccines via drinking water. A long-term study demonstrated that 6.7% of the analyzed water samples (n = 326) exceeded the recommended pH values for livestock [32].

Overall, the literature indicates that drinking water for livestock frequently exhibits pH variability influenced by source type, mineral composition, and seasonal factors. Although most samples fall within the recommended physiological range, deviations pose risks of system corrosion, reduced pharmaceutical stability, and potentially compromised animal performance. Although the AviPro™ *Salmonella* DUO vaccine is stable across a wide pH range, extreme values should be avoided. Therefore, it is strongly recommended that the pH value of the water be tested before vaccination. Consistent monitoring and control of pH levels throughout water supply systems is essential for maintaining water quality, safeguarding animal health, and ensuring food safety.

Water hardness is a measure of the concentration of dissolved minerals, primarily calcium and magnesium. Water hardness is typically categorized into three distinct ranges: soft (less than 8.4 °dH) (German degree of water hardness), medium (8.4 to 14 °dH), and hard (greater than 14 °dH). Calcium and magnesium ions have been shown to have a significant positive physiological effect; however, there are no precise guideline values for water hardness. Nevertheless, a high content of carbonate compounds can lead to the calcification of pipes and drinking equipment (filters, suppliers), and hard water should therefore be avoided [30]. Furthermore, the presence of magnesium ions at a concentration of 6000 mg/L has been observed to inhibit development and bone formation in immature chickens [83]. Additionally, magnesium and calcium salts in water can also interfere with sanitizers and cleaning equipment [84,85]. In the context of the examined *Salmonella* vaccine, the selected hardness ranges appear to exert minimal to no influence on the *Salmonella* vaccine levels. The tendency to form insoluble compounds is the main reason why water hardness is considered an important quality characteristic. Hard water can lead to the formation of biofilms and can reduce the cleaning power of certain products [86]. Consequently, in scenarios where hard water is present, a softening process can not only extend the lifespan of the pipe system but also indirectly contribute to the maintenance of animal health and may be necessary if antimicrobial substances are administered. However, the impact of softeners on vaccines is not known, and the use of water softeners in the presence of vaccine administration should therefore be avoided without further studies.

Dissolved ions are defined as charged particles (cations and anions) that are present in water as a result of natural processes, agricultural runoff, or industrial activity. Common ions include chloride (Cl^-), sulfate (SO_4^{2-}), nitrate (NO_3^-), nitrite (NO_2^-), and fluoride (F^-). The findings confirm that the AviPro™ SALMONELLA DUO vaccine is largely resilient to variations in common water ions. Fluoride, nitrate, and sulfate exerted minimal influence, supporting the vaccine's suitability across a range of environmental water conditions. Chloride was the only ion with a measurable, dose-dependent effect, yet even at the highest concentration tested, vaccine levels remained within manufacturer-specified limits. These results highlight the inherent robustness of the vaccine formulation, suggesting that routine fluctuations in water quality are unlikely to compromise efficacy. Nonetheless, since microbiological contamination of drinking water can also be a problem in livestock farming [32,87,88], the effect of high microbial content—or biofilm/biomass in general—on vaccines administered via water should be investigated further.

Disinfectants are able to inhibit the growth of microorganisms, such as bacteria and fungi, or exert a lethal effect on viruses [89]. Disinfectants can be categorized into two main groups: oxidizing and non-oxidizing disinfectants [90]. Oxidizing disinfectants are those that contain halogens like chlorine, iodine, and oxygen-releasing substances. Non-oxidizing disinfectants are those that bind to structures, such as quaternary ammonium compounds and amphoteric. The three most commonly used disinfectants for drinking water in European poultry housing are chlorine (HOCl/OCl^-), chlorine dioxide (ClO_2), and hydrogen peroxide (H_2O_2). In this study, all disinfectants examined were demonstrated to

be highly effective in deactivating the *Salmonella* vaccine within a very short period, even at low levels. This is not surprising as these disinfectants can be effectively used to disinfect drinking water and can also be used to destroy pathogenic microbes, including viruses, bacteria, and fungi [91].

Chlorine (Cl) is one of the most cost-effective and efficient disinfectants for drinking water and is therefore used worldwide. Free chlorine levels in poultry drinking water can result from the residues of previous disinfection and/or the sanitization of the water lines, as well as from high chlorine levels in the municipal water being used. Free chlorine levels in municipal water are limited to a maximum of 4 mg/L in the US, and the WHO recommends maximum levels of 5 mg/L. However, levels approaching 0.3 mg/L may already result in a noticeable chlorine taste. Although chlorine prevents the growth of harmful microbes, it also leads to the formation of disinfection by-products (DBPs) [92], which can be toxic or carcinogenic [93]. For this reason, European legislation limits their concentrations in drinking water [94]. In Germany, the regulations concerning drinking water set a maximum limit of 0.3 mg/L for chlorine [22]. In order to achieve effective disinfection, it is recommended that a minimum of 0.1 mg/L of free chlorine be maintained. However, in other countries such as the UK, chlorine spikes in municipal water can occur after a potential bacterial contamination. The present study indicates that even small amounts of free chlorine can impair the viability of vaccines, and that it is therefore important to monitor free chlorine levels, at least if drinking water vaccination is planned. However, a water stabilizer such as AviBlue™ provides effective protection up to free chlorine levels of 10 mg/L, which will be a helpful tool if free chlorine levels are high or unknown. As chlorine exhibits optimal disinfectant activity at a pH of 5–6 [95], and its efficacy is known to decrease at pH values above 8.5 [96], the potential negative effect of chlorine on the vaccine across different pH values should be evaluated further.

Chlorine dioxide (ClO₂) acts by disrupting the cellular processes of microorganisms, ultimately leading to their deactivation or destruction [97]. It is effective in removing biofilms, reducing odours, and controlling the growth of various pathogens. Chlorine dioxide is also known for its ability to penetrate and disinfect hard-to-reach areas, making it a popular choice for water treatment and disinfection in various industries, including poultry housing. In this study, it was shown that chlorine dioxide deactivates the vaccine very quickly, and it is therefore important to ensure that either the chlorine dioxide treatment of the water is stopped before vaccination or that a suitable water stabilizer is used.

Hydrogen peroxide (H₂O₂), another powerful disinfectant with broad-spectrum activity, works by releasing oxygen free radicals, which can damage the cell walls and membranes of pathogens, leading to their deactivation [98]. Hydrogen peroxide is known for its ability to penetrate organic matter and biofilms, making it useful in various disinfectant applications. Our study showed that hydrogen peroxide was able to quickly inactivate the live vaccine, even when used at a concentration that is safe for birds to drink; hence, it is critical to ensure that no traces of hydrogen peroxide are present during vaccination. The use of a water stabilizer did not protect the vaccine in this case, which is also relevant information for the farmer, as the use of a water stabilizer may provide a false sense of security in certain cases.

To increase the stability of live vaccines administered via drinking water, skimmed milk has been recommended [56]. However, the potential promotion of biofilm formation within drinking systems could lead to hygiene issues. Nowadays, the water stabilizer AviBlue™ is commonly used to buffer the effects of chlorine, metals, and cleaning agents in tap and borehole water, which can potentially reduce the viability of live vaccines, and thus protects the survival of live vaccines in the drinking water in a similar way to skimmed milk [99]. Active ingredients of AviBlue™ include carbonate/bicarbonate salts, sodium

thiosulphate and citric acid. Carbonate salts act as buffers by establishing an equilibrium between weak acids and conjugate bases, allowing them to neutralize added acids or bases. They maintain stable pH levels by consuming excess hydrogen ions or releasing them, essential for biological and environmental systems. When acid is added, carbonate or bicarbonate ions react with hydrogen ions to form bicarbonate or carbonic acid, preventing a drop in pH. When a base is added, carbonic acid or bicarbonate releases hydrogen ions to neutralize the OH^- , preventing a rise in pH. Furthermore, sodium carbonate, when added to hard water, precipitates out calcium and magnesium ions as their respective carbonates (CaCO_3 and MgCO_3), which are insoluble in water. This precipitation mechanism softens the water. Sodium thiosulfate neutralizes chlorine through a rapid redox (reduction–oxidation) reaction, breaking down oxidizing chlorine compounds into harmless chloride ions, sulfate, and water. It effectively removes both free chlorine and combined chlorine (chloramines) by acting as a reducing agent that consumes the oxidizing agent. Citric acid controls pH by releasing hydrogen ions to increase acidity, while acting as a powerful chelating agent that binds metal ions like Ca^{2+} , Mg^{2+} , and Fe^{3+} using its three carboxyl and hydroxyl groups. It forms stable, soluble complexes, removing these ions from solution to prevent scaling and improve cleaning, especially in acidic environments. It has previously been shown that water supplemented with AviBlue™ supports the survival of the AviPro™ SALMONELLA DUO live vaccine for at least 12 h—long enough to allow all birds, even very young ones, sufficient time to drink enough for successful vaccine uptake [100].

While in vitro studies offer valuable mechanistic insights under controlled conditions and reduce ethical concerns, their limited physiological relevance means that results must be interpreted with caution and validated through in vivo studies or field studies. Therefore, the results provide valuable guidance but should be verified under field conditions, including microbiological contaminations.

5. Conclusions

Overall, the results demonstrate that the AviPro™ SALMONELLA DUO vaccine exhibits high stability under a range of water quality parameters and moderate physiochemical conditions. Vaccine survivability was generally reduced under extreme conditions, such as high arsenic and iron concentrations or in the presence of hydrogen peroxide. These findings highlight the importance of maintaining appropriate water quality in drinking water lines to ensure optimal vaccine efficacy, while also indicating that AviBlue™ can only support stability under specific circumstances.

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