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Research Article

Influence of nanoemulsion on the adhesion and survival of *Aeromonas* sp. in meat and contact surfaces of meat

Einfluss der Nanoemulsion auf die Adhäsion und das Überleben von *Aeromonas* sp. in Fleisch und auf Kontaktflächen von Fleisch

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Summary

In the present investigation, coconut oil–based nanoemulsion, designated as AUSN3, was evaluated for its influence on the adhesion and survival of *Aeromonas* sp. AUBAS34 to beef, pork, and mutton and contact surfaces of meat such as polystyrene, glass, and stainless steel. AUSN3 treatment reduced the hydrophobicity, motility, biofilm formation, and bacterial adhesion of AUBAS34 to meat and the contact surfaces. AUSN3 treatment completely eliminated the AUBAS34 population in the contact surfaces of meat within a time period of 20 min. In meat surfaces, AUSN3 treatment resulted in 1.3–1.8 log reduction in *Aeromonas* population compared to sodium nitrite treatment and 2.4–3.2 log reduction compared to control.

Keywords: *Aeromonas* sp. AUBAS34, nanoemulsion, adhesion, meat, contact surfaces

Zusammenfassung

In der vorliegenden Untersuchung wurde die Behandlung mit Nanoemulsionen auf Kokosölbasis (AUSN3) auf ihren Einfluss auf die Adhäsion und das Überleben von *Aeromonas* sp. AUBAS34 (GenBank: KC253269.1) für verschiedene Fleischsorten wie Rindfleisch, Schweinefleisch und Hammel sowie für Kontaktflächen von Fleisch wie Polystyrol, Glas und Edelstahl evaluiert. Die AUSN3-Behandlung hat die Hydrophobizität, Motilität und Biofilmbildung verringert und die Bakterienadhäsion an Fleisch und Kontaktflächen von Fleisch verringert. Es wurde auch festgestellt, dass die Gesamtpopulationen der Verderbnisbakterien im Fleisch und auf den Kontaktflächen von Fleisch bei der AUSN3-Behandlung im Vergleich zur Behandlung und Kontrolle mit Konservierungsmitteln (Natriumnitrat) signifikant niedrig sind

Schlagworte: *Aeromonas* sp., Nanoemulsion, Adhäsion, Fleisch, Kontaktflächen

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1. Introduction

India ranks number one in world's milk production, accounting for 22% of the world's total milk production with an annual output of 186 million tons during the year 2019 (FAO, 2019). In spite of this, India ranks only eighth in the world's meat production, which vibes well with dairy industry, accounting for 4.9 million tons (Government of India, 2001; APEDA, 2008; FAO, 2016). An improvement in meat production in tune with the achieved milk production may serve to improve the economic conditions of the farming community, since 20.5 million people depend on livestock for their livelihood (Tamizhkumaran and Radhakrishnan, 2016).

The major key issues of concern in the Indian meat sector are the unhygienic conditions coupled with contamination/deterioration of meat (Government of India, 2001). In most of the underdeveloped and developing countries, including India, after slaughter meat is transferred to local retailers, it is held at an ambient temperature of 25 °C ± 2 °C for about 18–20 h and offered for sale in the retail shops (Kumudavally et al., 2008). This worsens the condition of meat, since holding of carcasses at ambient temperature for long hours results in microbial spoilage leading to undesirable changes in textural, physical, chemical, and microbiological parameters (Rao and Sreenivasamurthy, 1985).

Consumers' dissatisfaction over chemical additives in meat and meat products has led the food industry to seek for other alternatives that could minimize microbial spoilages and thereby extend the shelf life of fresh meat. Joe et al. (2012a) reported nanoemulsions as an alternative to antibiotics in enhancing the shelf life of fish stored at 20°C. These nanoemulsions are thermodynamically stable, transparent oil and water dispersions stabilized by an interfacial film of surfactant and co-surfactant molecules having a droplet size of less than 100 nm (Shafiq et al., 2007).

The constituents of nanoemulsions include emulsifiers and surfactants that are generally recognized as safe (GRAS) substances (Azmi et al., 2019). Nanoemulsions are used in the food industry for nutraceutical delivery, to impart food color and flavor, and as antimicrobials (Aswanthanarayan and Vittal, 2019).

Coconut oil is obtained from copra by a drying process, in which it is exposed to high temperature/sunlight until most of the moisture is removed (Nevin and Rajamohan, 2004). Coconut oil is preferred for cooking in the southern parts of India as frying oil due to its ability to withstand oxidation at higher temperatures (Prabhu, 2000).

Psychrotrophic *Aeromonas* sp. has been characterized as gram-negative, facultative anaerobes, which are rod-shaped and motile with polar flagella, and have been associated with the spoilage microflora of meat held at temperatures ranging from 10 °C to 20 °C (Ingram and Simonsen, 1980). These organisms predominantly cause spoilage if the temperature is not controlled well (Kraft, 1992). Moreover, *Aeromonas* sp., pathogens from fish and meat, are considered to be a major source of transmission of intestinal infections in humans (Praveen et al., 2016).

Studies pertaining to the control of *Aeromonas* sp. are of importance in the context of Indian sub-continent, where maintenance of cold chain is difficult due to power shortages, a routine affair since the demand for electricity is always more when compared to the supply.

Taking this into account in this study, we have studied the influence of coconut oil–based nanoemulsion on the adhesion of *Aeromonas* sp. to meat and contact surfaces of meat, followed by the evaluation on influence of nanoemulsion treatment on the bacterial counts in meat and contact surfaces.

2. Materials and methods

2.1. Nanoemulsion and *Aeromonas* strain

Coconut oil nanoemulsion (AUSN3) used in the study was prepared according to the methods of Hamouda et al. (1999) along with the required modifications that included the use of biosurfactant, instead of chemical surfactant (Joe et al., 2012b).

Aeromonas sp. was isolated from spoiled fish sample using Ryan's *Aeromonas* isolation base medium (Himedia, Mumbai, India), and the preliminary biochemical identification of the isolate was made based on Bergey's Manual of Systematic Bacteriology (Garrity et al., 2005). Molecular identification of the sequenced 16s ribosomal RNA was done based on the Ribosomal Database Project (RDP) (Wang et al., 2007). Phylogenetic tree was constructed using MEGA-X, with neighbor-joining (NJ) method having 1000 bootstrap replications, as described earlier (Tamura et al., 2011). The 16S rRNA gene sequence of *Aeromonas* sp. AUBAS34 was deposited in National Center for Biotechnology Information (NCBI) with the accession number KC253269.1.

2.2. Influence of the nanoemulsion on bacterial cell surface hydrophobicity, motility, and biofilm formation of AUBAS34

Cell surface hydrophobicity was studied by salt aggregation test (SAT) according to Jonsson and Wadström's (1984) protocol with 0.1–1.6 M ammonium sulfate. Biofilm formation was assayed based on a microtiter plate assay with AUBAS3 grown in biofilm-inducing medium comprising yeast peptone (YP) medium plus M63 minimal salt medium (Hossain and Tsyyumu, 2006) and measured based on the optical density (OD) levels of crystal violet present in the de-staining solution according to Djordjevic et al. (2002). Swarming assay was carried out in swarm plate (0.8% nutrient broth, 0.5% glucose, 0.5% agar) as per the protocol of Inoue et al. (2008). For SAT, biofilm formation, and swarming motility, 24-h grown bacterial cultures were centrifuged at 5000 rpm and the pellets were treated with AUSN3 and sodium nitrite at a volume of 10% v/v and at a concentration of 100 mg/L prior to the assays.

2.3. Surface disinfection and AUSN3 treatment on meat and contact surfaces of meat

Meat samples such as beef, mutton, and pork were obtained from the local slaughterhouses and sliced into equal pieces of 1 cm^3 volume. For meat surface disinfection, a combination of heat and mild organic acid treatment, as described earlier by Anderson et al. (1991) and Kumudhavally et al. (2008), was adopted. In brief, meat pieces were dipped in hot water (1:3 w/v) containing lactic and acetic acid (1% concentration each). The excess buffer in meat was drained and the meat was dried for 10 min under aseptic conditions. Thin sections of meat was made and observed under the microscope for any possible modification in the surface properties.

For AUSN3 treatment, meat samples were divided into three treatment groups including control. Group 1 samples were homogenized with a 50 ml solution containing 10% volume per flesh weight (v/w) of nanoemulsion (AUSN3); in group 2 samples, sodium nitrite was used as a preservative (Himedia Lab Private Limited, Mumbai, India) at a concentration of 100 mg/L, and in group 3, sterile distilled water was used as the control. To mimic different contact surfaces of meat used in our daily life, glass, polystyrene, and stainless steel balls of diameter 6 mm were selected for the study. Contact surfaces of meat (1 g) were surface sterilized in 0.2% (w/v) $HgCl_2$ for 3 min, followed by 70% ethanol for 1 min, and the residues were removed by 10 serial washings in sterile distilled water, irradiated for 15 min in a UV chamber, and maintained under strict aseptic conditions. Irradiation was made with two UV-C ultraviolet germicidal lamps of wavelength 254 nm and 40 W, and the specimen (meat and contact surfaces) was kept at a distance less than 30 cm from the UV light source. Surface of meat was sterilized by exposing to irradiation, followed by sterility checking by placing the samples in nutrient agar plates to test the efficiency of sterilization.

2.4. Adhesion assay

For adhesion experiments, *Aeromonas* sp. (1×10^3 Cfu/ml; initial load) was grown in nutrient broth (Himedia Lab Private Limited, India) comprising peptone 1.0 g per 100 ml, yeast extract 1.0 g per 100 ml, and NaCl 0.5 g per 100 ml for a period of 48 h. The bacterial culture suspension was then centrifuged at 7000 × g for 10 min, washed twice, and resuspended in 0.05 M potassium phosphate buffer at pH 6.1. The bacterial suspension was adjusted to a load of 1×10^7 Cfu/ml. For bacterial inoculation, 100 ml of bacterial suspension was poured into beaker (size 8 × 9 cm) containing different experimental groups as mentioned earlier and maintained at 20 °C. The suspension was decanted after 2 h of static incubation at 20 °C ± 2 °C and the contents were removed aseptically. For measuring the number of adhered bacteria, 1 g of sample was diluted with 10 ml of sterile peptone water (0.1% w/v) and homogenized for 1 min using a Teflon homogenizer (Remi Instruments, Mumbai, India). One milliliter of the sample was then serially diluted in phosphate-buffered saline (PBS) and plated in nutrient agar and the plates were incubated at 20 °C for 3 d.

2.5. Survival of *Aeromonas* in meat and contact surfaces of meat

For survival of *Aeromonas* sp. in meat and contact surfaces of meat, the bacteria were grown in nutrient broth for 24 h at 28 °C ± 2 °C. Bacterial cells were centrifuged at 5000 rpm for 10 min and suspended in phosphate buffer (pH 7.2) and adjusted to 1×10^7 Cfu/ml of bacteria. For bacterial inoculation, meat pieces and contact surfaces of meat were dipped in bacteria-containing buffer for 5 min, drained, and dried for 2 min under sterile conditions. All samples were packed in polythene bags which were then sealed airtight using a vacuum sealer. The whole experimental setup was maintained at 20 °C in an incubator in separate plastic boxes. Microbial analysis was done in triplicate at regular intervals up to a time period 60 h.

2.6. Statistical analysis

Statistical analyses were performed between different independent variables such as nanoemulsion, preservative, and control using one-way analysis of variance (ANOVA) with Tukey's honestly significant difference tests at a p value of 0.05. Minimum of three replications were maintained for each experiment, and the experiment was repeated twice to ensure the reproducibility of results.

3. Results and discussion

3.1. *Aeromonas* isolation and identification

The strain isolated through *Aeromonas* isolation medium was tentatively identified as *Aeromonas hydrophila* based on Bergey's Manual of Systematic Bacteriology. Phylogenic position of the isolated *Aeromonas* sp. AUBAS34 in comparison with other members of *Aeromonas* genus is given in Figure 1. Based on the RDP and NCBI Blast analysis of 16S rRNA gene, we propose the assignment of our strain as *Aeromonas* sp. AUBAS34. Meat can be infected with *Aeromonas* sp. due to poor hygienic conditions prevailing in the processing unit (Ogu et al., 2017) or due to washing of carcasses with contaminated water (Stratev and Odeyemi, 2016; Elbehiry et al., 2018). Toute and Murphy (1978) reported that *A. hydrophila* comprises 56% of the total flora associated with the spoiled chicken and it is present in various kitchen accessories and in work area.

3.2. Antibacterial activity of coconut oil and its nanoemulsion

Coconut oil used in our study did not demonstrate any antibacterial activity against *Aeromonas* sp. (data not shown). This lack of antibacterial activity of coconut oil against *Aeromonas* sp. is due to the presence of fatty acids like lauric acid in triglyceride form (Nitbani et al., 2016). Triglycerides and triglycerides do not possess any antibacterial activity, whereas monoglycerides show antimicrobial activity (Loung et al., 2014). Interestingly, when coconut oil was formulated as a nanoemulsion AUSN3, it showed bactericidal activity of 0.8% against *Aeromonas* sp., in which the bacterial population was completely eradicated at a time period of 20 min (data not shown). These results on the exhibition of antimicrobial activity when reduced to a nanoscale are comparable with the earlier findings of Hamouda et al. (2001), who reported effective bactericidal activity against *Bacillus cereus*, *Bacillus subtilis*, *Haemophilus influenza, Neisseria gonorrhoea*, *Streptococcus pneumonia*, and *Vibrio cholerae* by the nanoemulsion 8N8 in 15 min.

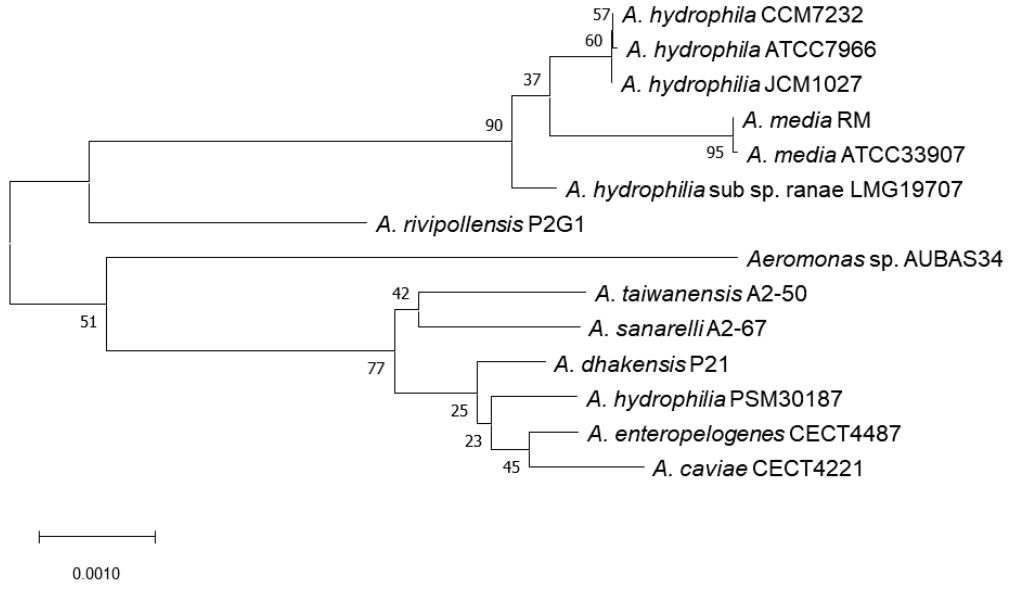


Figure 1. Phylogenic analysis of the isolated strain *Aeromonas* sp. AUBAS34 along with its neighbours with a bootstrap value of 1000
Abbildung 1. Phylogene Analyse des isolierten Stammes Aeromonas sp. AUBAS34 zusammen mit seinen Nachbarn mit einem Bootstrap-Wert von 1000

3.3. Hydrophobicity, biofilm formation, and motility influenced by nanoemulsion

Reduction in hydrophobicity, biofilm formation, and swarming motility in AUBAS34 was observed on AUSN3 treatment and the results are given in Table 1. Cell surface hydrophobicity of *Aeromonas* sp. was measured based on SAT value. AUSN3 treatment recorded a SAT value >1.6, while the preservative and control recorded SAT values of 1.2 and 0.8, respectively. Swarm motility of 17.6%, 29.6%, and 34.9% was observed for AUSN3, preservative, and control, respectively. OD values of 0.54, 0.94, and 1.42 for biofilm formation were observed with AUSN3, preservative, and control, respectively, at 595 nm.

Results on the reduction of hydrophobicity and motility as influenced by nanoemulsion application are in accordance with our previous finding (Joe et al., 2015) that surfactin-based nanoemulsion is able to reduce the swarming motility and hydrophobicity in *Pectobacterium carotovorum* strains. Reduction in biofilm formation by nanoemulsion application is in line with the reports of Ramalingam et al. (2012), who reported reduction in biofilm and planktonic forms of *Actinomyces viscosus, Candida albicans, Streptococcus mutans*, and *Lactobacillus casei* strains. The reason may be due to the interaction of cationic charged emulsion with the anionic charge of the bacterial cell wall, affecting motility, hydrophobicity, and biofilm formation (Hamouda and Baker, 2000).

3.4. Adhesion of *Aeromonas* to meat and contact surface of meat

Influence of AUSN3 treatment on the adhesion of *Aeromonas* sp. to meat and contact surfaces of meat was studied and the results are presented in Table 2.

AUSN3 treatment resulted in 1.4, 0.8, and 1.5 log reduction in *Aeromonas* population in mutton, beef, and pork samples, respectively, compared to control. Similarly, AUSN3 treatment in mutton, beef, and pork samples recorded 0.6, 0.4, and 0.8 log reduction in *Aeromonas* population compared to treatment with preservative. So far, there has been no study on the influence of nanoemulsion on the microbial adhesion to meat surfaces. However, the anti-adhesive property of the nanoemulsion could be explained based on the presence of biosurfactant molecules in nanoemulsion formulation. De Araujo et al. (2014) reported the anti-adhesive property in biosurfactants that could enable removal/reduction of bacterial biofilms on food surfaces. Roy (2017) reported that biosurfactants could change the hydrophobicity of surfaces, which in turn can influence the surface bond with microbes.

AUSN3 treatment in polystyrene, glass, and stainless steel surfaces recorded 1.8, 1.9, and 2.5 log reduction in *Aeromonas* population compared to control, whereas in comparison with the group treated with preservative, AUSN3 recorded 1.1, 1.5, and 2.3 log reduction of *Aeromonas* population in the contact surface of meat. Previous reports of Ramalingam et al. (2012) reported that adhesion of planktonic *S. mutans*, *L. casei*, *A. viscosus*, *C. albicans*, and mixed culture of these strains on the glass surface was reduced up to 99.5% in nanoemulsion-treated groups, when compared to controls. Pompilio et al. (2008) reported hydrophobicity as a significant determinant for adhesion and biofilm formation of *Stenotrophomonas maltophilia* on the polystyrene surfaces, and similar results of reduction in hydrophobicity and biofilm formation due to nanoemulsion treatment were obtained in this study.

3.5. Survival of *Aeromonas* species in meat and contact surface of meat

Results on the influence of AUSN3 treatment on the total bacterial population of different meat and contact surfaces of meat are presented as log Cfu/g in Figure 2a–f. No detectable *Aeromonas* population was observed in the contact surfaces of meat, such as polystyrene, glass, and stainless steel, treated with AUSN3 at a sampling period

Table 1. Influence of nanoemulsion treatment on the hydrophobicity and biofilm formation of *Aeromonas* sp.
Tabelle 1. Veränderungen der Hydrophobizität und Biofilmbildung von *Aeromonas* sp. durch die Nanoemulsionsbehandlung

Treatment	Hydrophobicity (SAT)	Swarming motility % surface coverage	Biofilm formation (OD 595 nm)
AUSN3	>1.6	17.5 ± 2.4c	0.54 ± 0.18c
Preservative	1.2	29.6 ±3.2b	0.94 ± 0.12b
Control	0.8	34.9 ± 2.7a	1.42 ± 0.22a

Hydrophobicity determined according to SAT (salt aggregation test). Biofilm formation using microtiter plate assay. Swarming motility in the presence of 0.5% agar. Different letters after values indicate that there is a significant difference at $p < 0.05$.

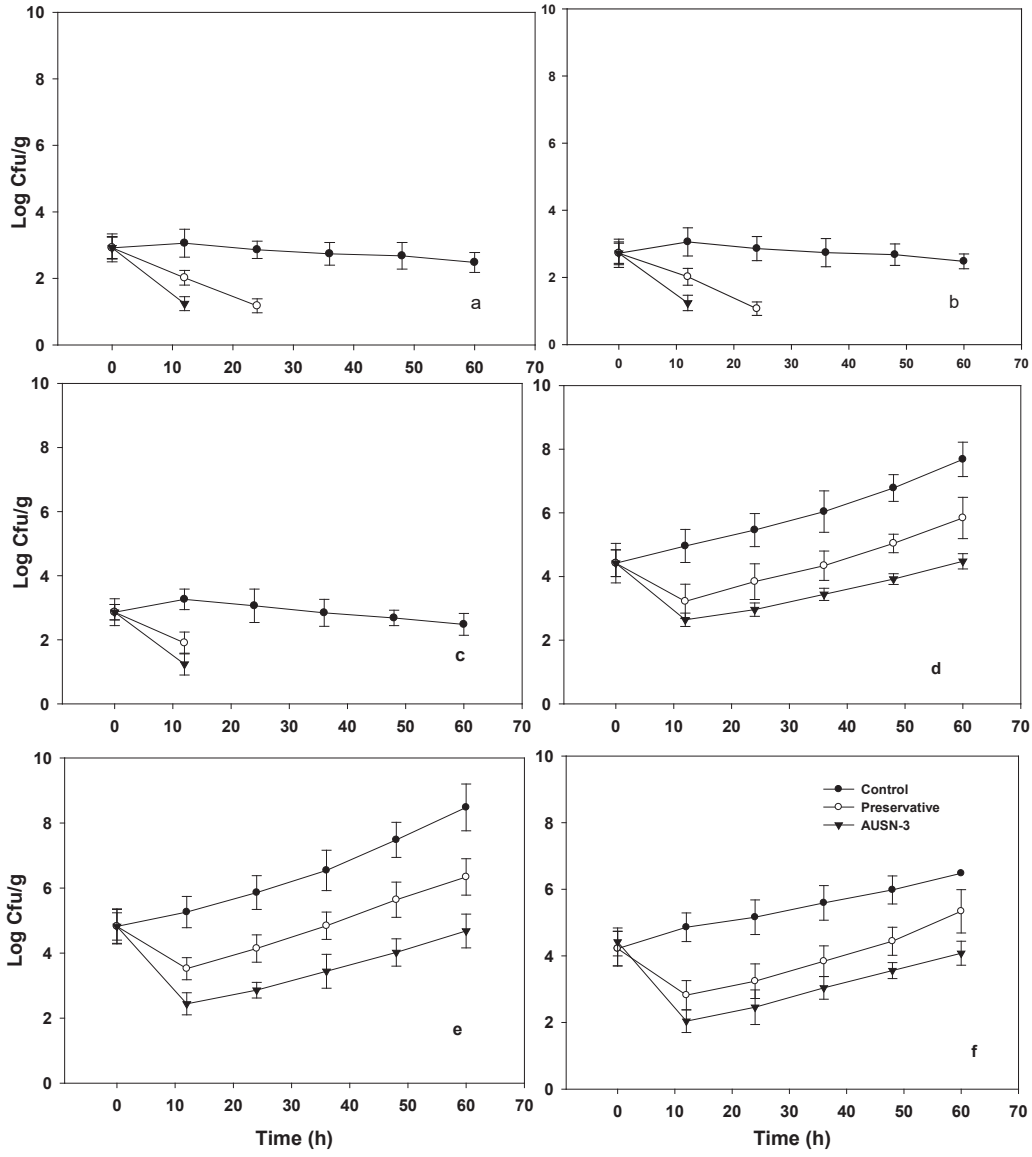


Figure 2. Influence of AUSN3 on the survival of *Aeromonas* sp. in meat and contact surfaces of meat at 20 ± 2 °C. a) polystyrene, b) glass, c) stainless steel, d) beef, e) pork and f) mutton. Values are a means of three replications ± standard deviations.
Abbildung 2. Einfluss von AUSN3 auf das Überleben von *Aeromonas* sp. in Fleisch und Kontaktflächen von Fleisch bei 20 ± 2 ° C. a) Polystyrol, b) Glas, c) Edelstahl, d) Rindfleisch, e) Schweinefleisch und f) Hammel. Die Werte sind Mittelwerte aus drei Wiederholungen ± Standardabweichungen.

of 20 min. Whereas in mutton, at the end of the sampling period of 60 h, 3.2 log reduction in *Aeromonas* population with respect to control and 1.8 log reduction with respect to preservative were observed with AUSN3 treatment. Similar trend was observed with the *Aeromonas* population of AUSN3-treated beef, which recorded 2.8 log reduction and 1.7 log reduction with respect to control and preservative treatment, respectively. In AUSN3-treated pork, 2.4 log reduction in *Aeromonas* population in the control and 1.3 log reduction in *Aeromonas* population in the preservative were observed. Recently, Swathy et al. (2018) reported the use of neem oil–based nanoemulsion against *Aeromonas culcicola A. culcicola* as an alternative for synthetic antibiotics in fish. Results of the present study on the influence of nanoemulsion treatment on the bacterial population of different meat and contact surfaces of meat are in accordance with the report that oil in water microemulsion was active against food-borne pathogens including

Table 2. Influence of AUSN3 treatment on the adhesion of *Aeromonas* sp. to meat and contact surfaces of meat
Tabelle 2. Einfluss der AUSN3-Behandlung auf die Adhäsion von *Aeromonas* sp. zu Fleisch und Kontaktflächen von Fleisch

Cultivation media	Treatment	Number of cells adhered (log CFU/g)*					
		Meat surface			Contact surface of meat		
		Beef	Mutton	Pork	Polystyrene	Glass	Stainless steel
PCA agar	AUSN3	5.8 ± 0.03^{c}	5.5 ± 0.11^{b}	5.6 ± 0.04^{c}	4.3 ± 0.10^{c}	3.4 ± 0.0^{b}	3.2 ± 0.1^{b}
	Preservative	6.4 ± 0.04^{b}	5.9 ± 0.08^{a,b}	6.4 ± 0.10^{b}	5.2 ± 0.08^{b}	4.9 ± 0.4^{a}	5.5 ± 0.1^{a}
	Control	7.2 ± 0.10^{a}	6.3 ± 0.44^{a}	7.1 ± 0.12^{a}	6.1 ± 0.10^{a}	5.3 ± 0.1^{a}	5.7 ± 0.0 ^{a}

*No. of bacteria that adhered after a time period of 2 h at 20 °C. Different letters after values indicate that there is a significant difference at $p < 0.05$.

Staphylococcus aureus, *Escherichia coli, Pseudomonas aeruginosa*, *Salmonella typhimurium,* and *Listeria monocytogenes* (Teixeira et al., 2007). Reduction in survival of *Aeromonas* sp. in meat and contact surfaces of meat may be due to the reduction in hydrophobicity, motility, and biofilm parameters observed in this study on AUSN3 treatment and also due to decrease in adhesion of *Aeromonas* sp. to meat and contact surfaces of meat.

4. Conclusions

In the present study, AUSN3 treatment reduced the bacterial hydrophobicity, biofilm formation, and adhesion to meat and contact surfaces of meat. The bacterial populations were found to significantly reduced by AUSN3 treatment when compared to preservative and control treatments. This nanoemulsion could be used as an alternative protective agent in preventing the adhesion of *Aeromonas* sp. and other related strains to food and contact surfaces of food. This prevention of microbial adhesion to meat may help us to prevent microbial spoilage of meat and meat products.

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