

Original Research Article

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Biocides Resistance Profiles of Biofilm Forming Bacteria of Dairy Niche and their Control

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ABSTRACT

Microbial biofilms are of special concern to dairy and food industry, as biofilms on raw materials or food contact surfaces represent possible sources of contamination with spoilage or pathogenic microbes. The present study was carried out to evaluate biocide resistance profiles of biofilm forming bacteria of dairy niche towards commonly used biocides and their control in biofilm formation. Genetically confirmed 17 isolates of genera *Bacillus*, *Staphylococcus*, *Salmonella*, *Pseudomonas*, *Listeria* and *E. coli* were evaluated for their biocide resistance profiles by determining their minimum inhibitory concentration (MIC) and also microbial biofilm eradicating concentration (MBEC) against commonly used biocides (iodophore, sodium hypochlorite, and benzalkonium chloride) in dairy/food industry. The *Listeria*, *Salmonella* and *Staphylococcus* exhibited highest MBEC against iodophore (550 ppm), *Listeria* against sodium hypochlorite (550 ppm); *Bacillus*, *Pseudomonas*, *Listeria* and consortia against benzalkonium chloride (650 ppm). Benzalkonium chloride and sodium hypochlorite were found most efficacious against all tested isolates and their consortia as they could reduce higher log counts after treatment in comparison to iodophore.

Keywords

Biofilm, Irreversible, Efficiency, Resistance, Persistence

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Introduction

Bacteria irreversibly attached to a substratum and embedded in an extracellular polymeric matrix that confers resistance for the involved microorganisms of their own synthesis forming a community of microorganisms is known as the biofilm (Donlan, 2002). Generally, biofilm maturation occurs in five distinguished phases: reversible adsorption, irreversible attachment, maturation I, maturation II and dispersion. Now, second

phase of biofilm formation is irreversible attachment wherein bacteria adhere firmly to substrate surface and lose their mobility. Bacterial cells attach to each other and to the substrate surface and thus formation of bacterial micro colonies begins. This phase lasts for two hours (Maric and Wranes, 2007).

Microbial biofilms which form on all types of surfaces of technological systems in the dairy industry and on dairy farms adversely affect the quality and safety of final products, i.e.

both foodstuffs and raw materials used for their production as besides bacteria insignificant to health, they may contain pathogenic microorganisms also. Thus, direct contact with raw materials or foodstuffs can cause secondary contamination due to which the product will become unsafe. Bacterial contamination that causes food decay and decreased quality is technologically important because of the fact that a number of microorganisms are alimentary pathogens, e.g. *Staphylococcus aureus* or *Listeria monocytogenes* and some others make a serious problem directly affecting human health when present in the food.

Outbreaks of pathogens associated with biofilms have been related to the presence of *Listeria monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Salmonella spp.* *Staphylococcus spp.* and *Escherichia coli* O157:H7 (Aarnela *et al.*, 2007). Pathogenic microorganisms (from genera *Bacillus*, *Streptococcus* and *Staphylococcus*, *Shigella*, *Escherichia*, and *Enterobacter aerogenes*) participated in the biofilm formation on the surfaces of a post-pasteurisation unit in a dairy plant. Several studies have reported the persistence of pathogenic bacteria on food and on medical equipments despite the use of cleaning and disinfection procedures (Brooks and Flint 2008; Simoes *et al.*, 2010; Srinivasan *et al.*, 2003). Poulsen (1999) reported that persistence of biofilms is probably due to the failure of disinfectant products, the inappropriate disinfection protocols and increased tolerance of bacteria due to adaptation to antimicrobial agents.

Biofilms are usually formed by various species of microorganism, which protect each other against the effects of biocidal (antibacterial) agents and are resistant to these agents. This is caused by the different resistance of respective microbial species

against the agents used (Wirthlin *et al.*, 2005). Sanitation regime, which meets the basic requirements, must be based on the knowledge of the particular conditions of the sanitised environment: (1) detected pathogens, colonising the cleaned surface; (2) knowledge of the aimed effects of the selected biocides against the pathogens; (3) chemical and physical properties of the surfaces; (4) hygienic design of technological systems (Salo *et al.*, 2001). To control biofilm formation and its eradication, various strategies have been explored in dairy/food industry that includes of incorporation of biocides such as benzalkonium chloride (BKC), sodium hypochlorite (SH) and iodophore (ID) etc. in CIP system which is one of the most common strategies.

Most efficient strategy for actions against biofilm to ensure food safety and quality in the dairy food industries could be to clean and disinfect food contact surfaces before bacteria get attached firmly to these surfaces to form mature biofilms. Hence it follows that it is necessary to test first the effectiveness of the sanitation agents and sanitation procedures under model laboratory conditions, above all in the dairy industry and on farms. Therefore, present investigation was aimed to evaluate biofilm formation potential of pathogenic and spoilage bacteria of dairy niche as well as determination of their biocide resistance profile to emerge with better cleaning strategies to enhance food safety and quality and to control them.

Materials and Methods

Bacterial cultures

A total of 24 bacterial cultures including their standard strains were used in the present investigation. The 17 isolates belonging to six genera *viz.*, *Escherichia coli*, *Pseudomonas*, *Salmonella*, *Bacillus*, *Listeria* and

Staphylococcus were identified from biofilms of dairy niche points.

Determination of biofilm formation potential by biomass indicator staining method

Quantitative biofilm assay was performed by using crystal violet staining method using a 96 well microtitre plate. Briefly, all isolates were grown overnight in trypticase soy broth (TSB), inoculated at 1% inoculum in 200 μ l of the same medium and plates were incubated at 37°C/24 h. Quantitative estimation of biofilm formation was determined by the method Stepanovic *et al.*, (2007): non biofilm producer = $OD \leq OD_C$; weak biofilm producer = $OD_C < OD \leq 2 \times OD_C$; moderate biofilm producer = $2 \times OD_C < OD \leq 4 \times OD_C$; strong biofilm producer = $4 \times OD_C < OD$. Subsequently on the basis of optical density, isolates were categorized as strong, moderate, weak and non-biofilm formers.

***In-vitro* Biofilm Development on Stainless Steel chips**

The method of Milanov *et al.*, (2009) was used with slight modifications for *in-vitro* biofilm development. *In-vitro* biofilm was developed on stainless steel (SS) chips of size 1×1×0.2 cm. The SS chips for biofilm development were first treated by keeping in 1 N HNO₃ solution for 30 minutes to clean it properly and then autoclaved a 121°C at 15 PSI for 15 min. The microbial cell concentrations of overnight grown cultures in TSB were adjusted to OD of 0.5 McFarland standards which correspond to 10⁸ CFU/ml. The individual SS chips were placed in each well of 6-well plate. The cell suspension of test strain was added @ 1% into each well containing fresh 9 ml TSB. These experiment was divided into two sets: set I (incubated at 37°C/3 h) and set II (incubated at 37°C/48 h). After incubation at 37°C for 3 h and 48 h,

chips were removed and washed with 3 ml of phosphate buffer saline (PBS) to remove loosely attached cells and nutrients. The biofilm were scraped off from SS chips, by moving a sterile swab approximately 10 times over the biofilm area. The swab was then placed into a tube containing 5 ml of physiological saline and vigorously vortexed for 2 minutes. Appropriate dilutions were prepared and plated out on nutrient agar for standard plate count.

Determination of minimum inhibitory concentration (MIC) and microbial biofilm eradicating concentration (MBEC)

The MIC and MBEC assay were carried out as per the following protocol. Respective stock solutions of benzalkonium chloride (10⁶ ppm), sodium hypochlorite (10⁴ ppm), and iodophore (10⁴ ppm) were appropriately diluted in tryptic soy broth (TSB) tubes to obtain required concentrations (0 to 400 ppm in steps of 50 ppm for determination of minimum inhibitory concentration). Aliquots of 1 ml TSB containing different biocides concentrations, were inoculated at 1% inoculum rate with overnight grown cultures and incubated at 37°C/24 h. Throughout the experiment, TSB tubes (without biocide) inoculated with respective cultures were used as positive controls, whereas un-inoculated TSB tubes (with biocides) as negative controls (Ac). After incubation, 200 μ l aliquots (in triplicate) of respective cultures were transferred to a fresh 96 microtitre well-plate and optical density at 620 nm was measured using microplate reader (Spectrostar-Nano). Inhibitory concentration end point was considered as lowest concentration of biocide at which absorbance of test (A) was less than or equal to that of un-inoculated control (Ac). The efficiencies of biocides (100-650 ppm in steps of 50 ppm) against biofilms of selected isolates were determined in terms of microbial biofilms eradication concentration (MBEC).

To determine MBEC, contents of microtitre plate with 24 h old biofilm were decanted and treated with biocides for contact time of 30 min. Neutralizing solutions used were 0.5% sodium thiosulphate in phosphate buffer (pH 7.2) in case of sodium hypochlorite and iodophore, and tween lecithin (stock solution: 40 gm L- α lecithin and 0.5 ml Tween-80 per litre working solution: 25 ml of stock solution diluted 500 ml in distilled water) in case of benzalkonium chloride. Neutralizing solutions were added to stop the activity of biocide after pre-determined contact of time. The plates were then decanted, rinsed gently with sterile water and stained using crystal violet. The bound dye was resolubilized in 33% glacial acetic acid, absorbance of which was read as described earlier. MBEC was considered as the concentration of biocide at which $A \leq A_c$.

Efficacy evaluation of biocides on biofilm formation

Biofilm were developed on SS chips as per Milanov *et al.*, (2009) for individual selected strains of *E. coli*, *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Listeria*, and *Salmonella* and their consortia for pre-decided incubation time (0, 0.5, 1, 1.5 and 2 h), which indicates of irreversible stage of biofilm formation. After incubation the chips were washed with 3 ml of phosphate buffer saline (PBS) to remove loosely attached cells and nutrients. Biofilm forming bacteria were scraped off from SS chips by moving a sterile swab approximately 10 times over the biofilm area.

The swab was then placed into a tube with 5 ml of physiological saline and vigorously vortexed for 2 minutes. Appropriate dilutions were prepared and plated out on standard plate count on nutrient agar for viable bacterial counts to be considered as control counts without giving any biocide treatment at each specific incubation time. The treatment with biocides were performed as the whole surface

of SS chips were covered with the specified biocides (iodophore, sodium hypochlorite and BKC) with MBEC confirmed concentration, (Table 2), for contact time of 30 min after each specific incubation times of irreversible stage as prescribed earlier. After treatment biocides were removed and their effect was inhibited using appropriate neutralizing solutions as mentioned earlier. Biofilm bacteria were scraped off from SS chips by moving a sterile swab approximately 10 times over the biofilm area.

The swab was then placed into a tube with 5 ml of maximum recovery diluent and vigorously vortexed for 2 minutes. Appropriate dilutions were made in sterile physiological saline, and the numbers of cells were determined by standard plate count on nutrient agar. The counts of control and counts after treatment were observed to check the biocides efficiency at different incubation time of irreversible stage for each target genera and their consortia biofilm.

Statistical analysis

The statistical analysis of data was carried out using SAS 9.3 (SAS Institute Inc., Cary, USA). Results were analysed and the significance level was calculated using Tukey's Studentized Range (HSD) test and results are considered significant at ($p < 0.05$). The results were expressed as mean \pm standard deviation.

Results and Discussion

Determination of biofilm formation potential by biomass indicator staining method

A total of 24 cultures including six standard cultures and their consortia (comprising of one isolate of each genera) were checked for their biofilm formation potential. It was assayed by

using 96 well polystyrene microtitre plate and indirect quantitation was carried out using crystal violet staining. The isolates and standard cultures were categorized into no (-), weak (+), moderate (++) and strong (+++) biofilm formers as per the method of Stepanovic *et al.*, (2004).

Amongst the tested strains, higher biofilm formation potential was observed for two *Staphylococcus*, two *Pseudomonas* and one *Listeria* isolates. Two of *Salmonella* isolates were designated as weak biofilm producers. Two isolates of *E. coli* and one of *Bacillus* showed moderate biofilm formation potentials.

Biofilm formation potential on stainless steel chips

Cell counts were determined after incubation period of 3 and 48 h at 37°C. The observations on biofilm formation potential of isolates on SS chips are displayed in Table 1. The number of cells on stainless steel chips ranged from 10^3 to $10^6 \log_{10}$ CFU/cm² after 3 h of incubation and 10^5 to $10^8 \log_{10}$ CFU/cm² after 48 h of incubation at 37°C. The results are in corroboration with the research findings of Chae and Schraft (2001), who reported similar values for attachment of *L. monocytogenes* cells, on glass surface for 3 h incubation at 37°C.

Among the entire target microflora, significantly higher biofilm formation potential, in terms of \log_{10} CFU/cm² was observed for *Pseudomonas*. Our findings in this regard are in accordance with the previous study by Klausen (2003), where *Pseudomonas* cultures exhibited strong biofilm formation owing to secondary attachment. Strain *Staphylococcus* (PC1 ST 4) had a lower attachment on SS chips after 3 h of incubation i.e. $3.23 \pm 0.132 \log_{10}$ CFU/cm², which increased after 48 h incubation to $7.857 \pm 0.174 \log_{10}$ CFU/cm² at 37°C. In case of

Pseudomonas (PC2 Ps 3), the counts were $5.19 \pm 0.41 \log_{10}$ CFU/cm² after 3 h which to $7.965 \pm 0.174 \log_{10}$ CFU/cm² after 48 h incubation. These results are in accordance with the investigations of Chae and Schraft (2000), who experimentally proved that growth of *L. monocytogenes* in biofilm were independent of initial attachment. High biofilm formation potential on SS chips was observed for consortia after 3 h incubation as compared to individual strains, which showed almost two times higher log counts.

Determination of inhibitory concentration of biocides against target strains

The standard cultures along with one tested isolate from each chosen genera were selected for determination of their control on biofilm formation as per Table 2. The MIC and MBEC of dairy niche isolates against iodophore was observed in the range of 20-70 ppm and 250-550 ppm, respectively for target genera and their consortia. The MIC and MBEC values for sodium hypochlorite ranged from 100-200 ppm and 300-550 ppm, respectively for tested strains and their consortia. Our results regarding MBEC of sodium hypochlorite are in agreement with Souza *et al.*, (2014) who stated that concentration of 250 mg/L of SH was not sufficient for complete removal of the cells of *S. aureus* from a biofilm on polypropylene and stainless steel surfaces. The MIC and MBEC values in case of benzalkonium chloride ranged from 150-250 ppm and 500-650 ppm, respectively. Results on MIC, recorded that *E. coli* was highly susceptible and *Pseudomonas* most resistant against benzalkonium chloride. Our results are in agreement with Gilbert and Moore, (2005), who also noticed that some species of bacteria, notably *Pseudomonas aeruginosa*, are relatively insensitive to QAC biocides and it is thought to be related to a failure of compounds to penetrate the outer membrane.

Table.1 Biofilm formation potential on SS chips of selected isolates of dairy niche

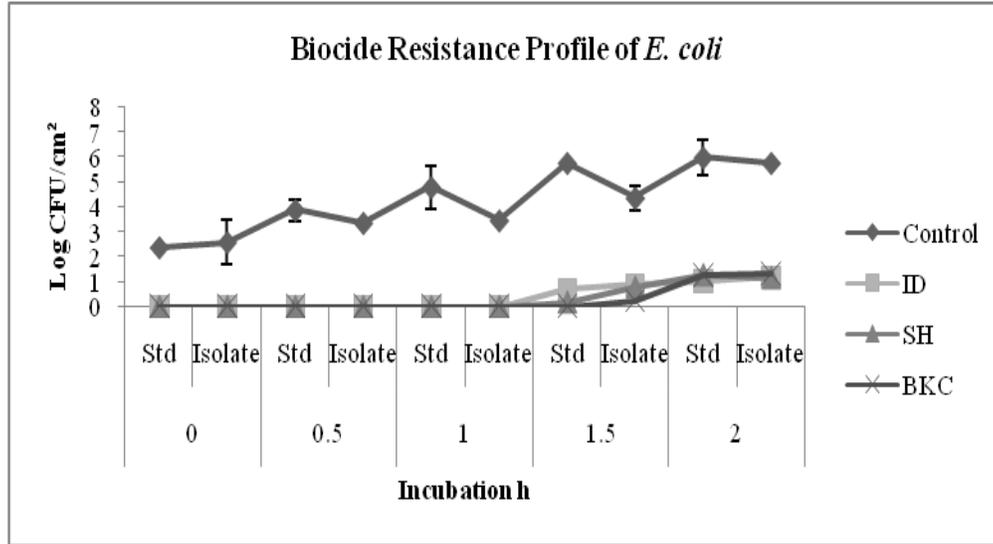
S. No.	Genera	Log ₁₀ CFU/cm ² at 37°C (mean value ± SD)	
		after 3 h incubation	after 48 h incubation
1	<i>Bacillus</i>	3.23±0.28	6.17±0.20
2	<i>Listeria</i>	3.34±0.075	6.345±0.132
3	<i>Pseudomonas</i>	5.19±0.41	7.857±0.23
4	<i>Staphylococcus</i>	3.23±0.132	7.965±0.174
5	<i>E. Coli</i>	3.34±0.162	6.85±0.29
6	<i>Salmonella</i>	3.73±0.25	5.85±0.28
7	Consortia	6.65±0.34	8.231±0.325

Table.2 MIC and MBEC of biofilm forming microbes of dairy niche

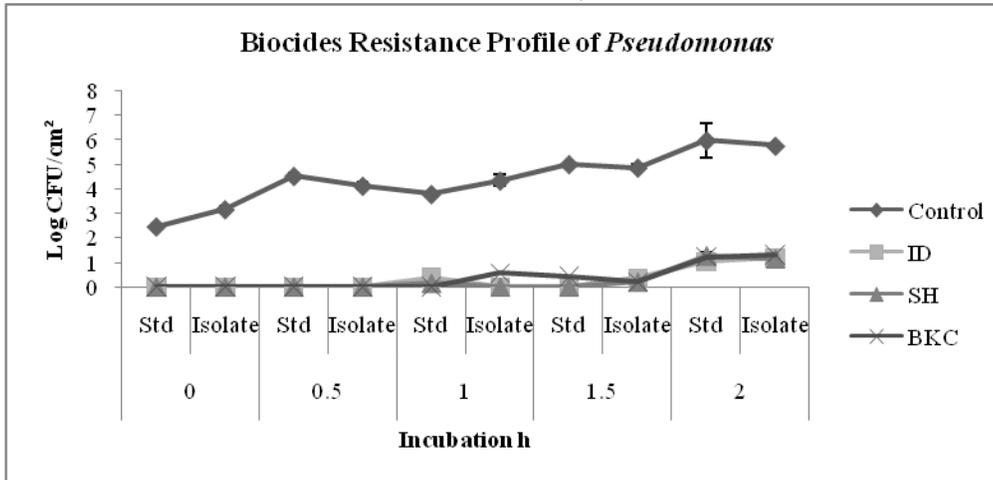
Genera/isolate		MIC/MBEC of biocides (ppm)					
1. Bacillus							
Standard Culture	Selected isolates	Iodophore		Sodium hypochlorite		Benzalkonium chloride	
		MIC	MBEC	MIC	MBEC	MIC	MBEC
	<i>B. cereus</i> ATCC 11778	20	350	200	400	200	650
	ST B 1	30	300	250	450	250	650
2. Pseudomonas							
	<i>Ps. aeruginosa</i> MTCC 741	20	300	200	450	250	650
	PC2 Ps 3	30	300	250	450	250	600
3. E. coli							
	<i>E. coli</i> ATCC 25922	20	250	100	300	150	500
	PC2 EC 3	20	250	150	300	150	500
4. Listeria Monocytogenes							
	<i>L. monocytogenes</i> ATCC 19115	70	550	150	550	200	650
	PC2 Lis 3	70	550	150	550	200	600
5. Salmonella							
	<i>S. typhi</i> NCDC 113	70	550	150	500	150	500
	PC1 S 3	70	550	150	500	150	500
6. Staphylococcus							
	<i>S. aureus</i> MTCC 3160	30	550	125	450	150	600
	PC1 ST 4	40	600	150	450	200	650
6. Consortia							
	Consortia	20	350	150	350	250	650

Consortia: Mixture of each selected genera isolate in same proportion, Sampling points: (PC1:Pre-chiller, PC2:Post-chiller, ST: Storage tank valve, PO: Pasteurizer outlet), ppm: parts per million.

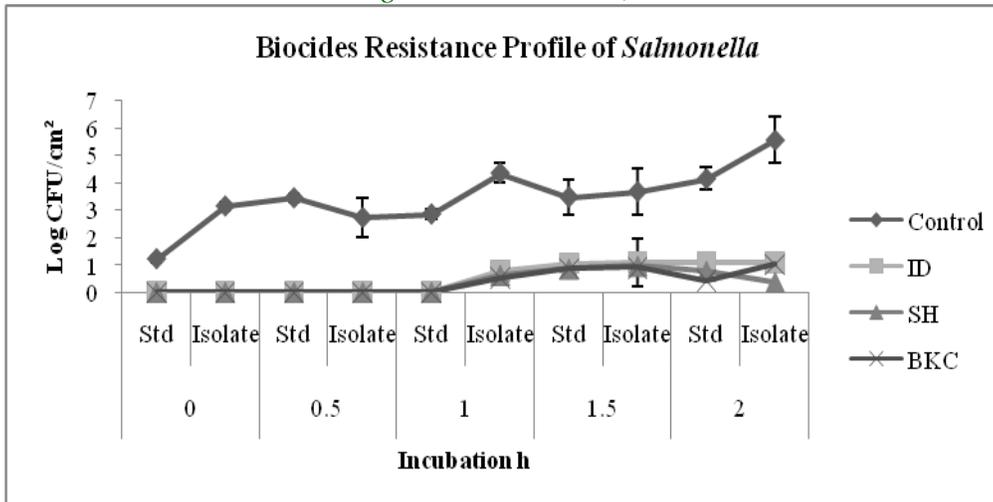
Fig.1 Efficacy of biocides in controlling biofilms on stainless steel chips



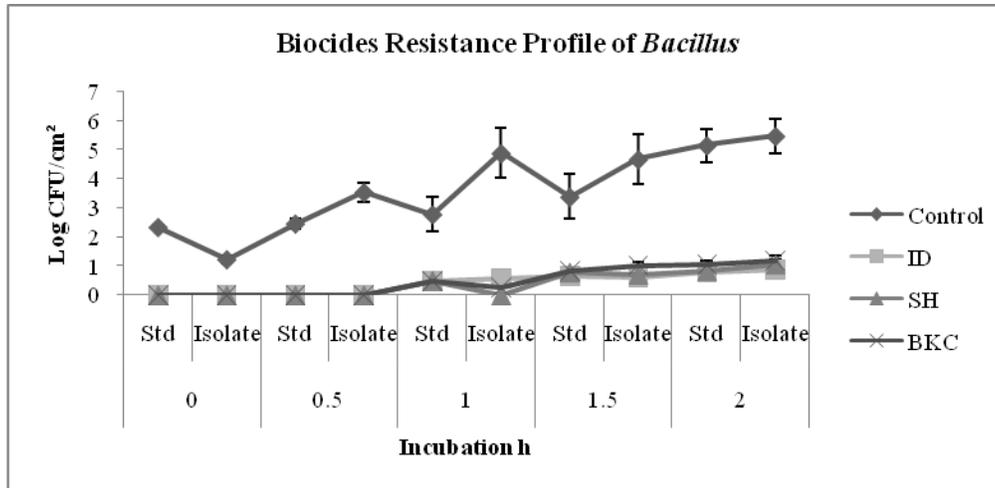
i. **STD: *E. coli* ATCC 25922; ISOLATE: PC2 EC 3**



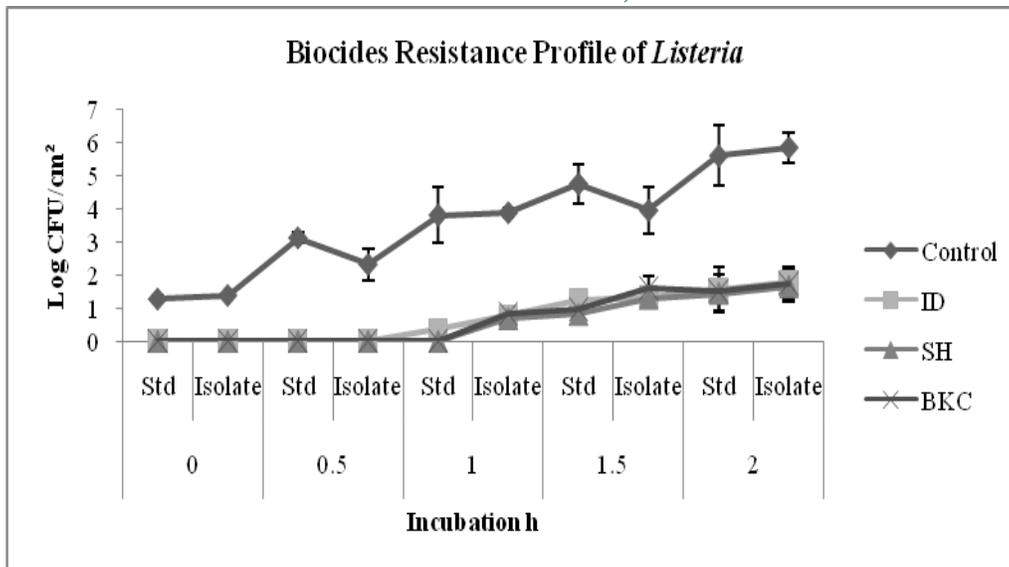
ii. **STD: *Ps. aeruginosa* MTCC 741; ISOLATE: PC2 PS 3**



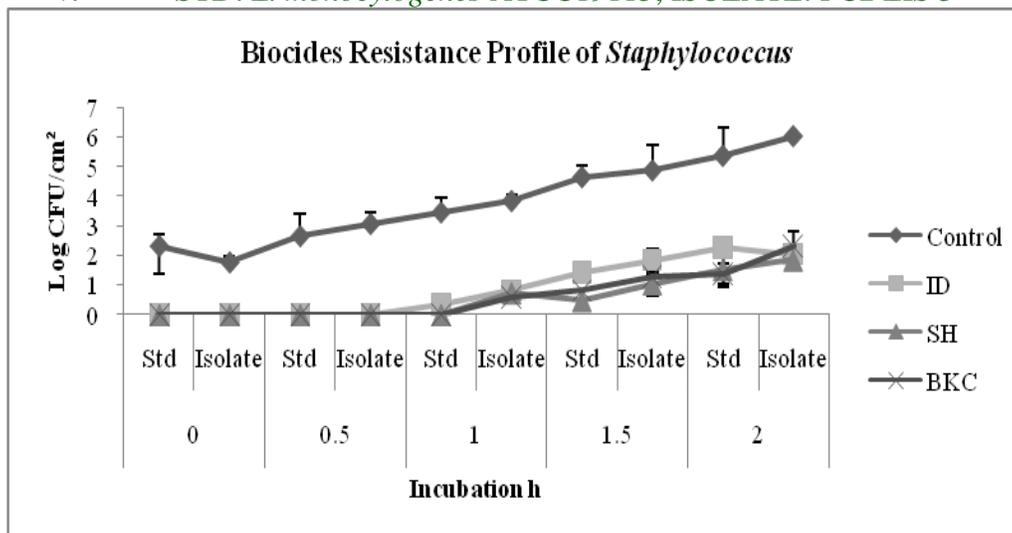
iii. **STD: *S. typhi* NCDC113; ISOLATE: PC1 S 3**



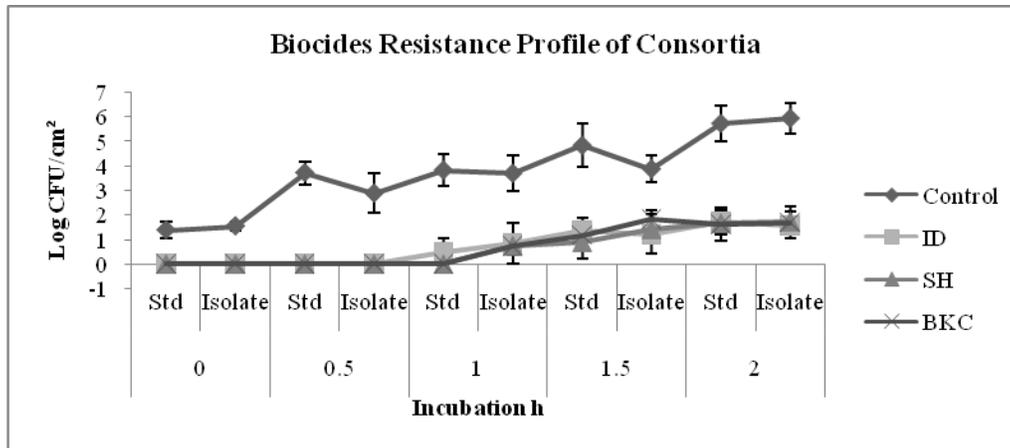
iv. STD: *B. cereus* ATCC 11778; ISOLATE: ST B 1



v. STD: *L. monocytogenes* ATCC19115; ISOLATE: PC2 LIS 3



vi. STD: *Staph. aureus* MTCC 3160; ISOLATE:PC1 ST 4



vii. **STD: Mixture of standard strains shown in above graphs; isolate: mixture of isolated strains shown in above graphs**

ID: Iodophore (grey column), SH: Sodium Hypochlorite (dark grey column), BKC: Benzalkonium Chloride (black column), control (ash column). Effect of these disinfectants on biofilms of Std: standard culture and Isolate: selected strain grown on stainless steel chips of respective genera (shown as the graph head) and their consortia, at 0, 0.5, 1.0, 1.5 h. Data represent the mean of recovered viable and culturable cells count ($\log \text{CFU}/\text{cm}^2$) \pm SD. Consortia: Mixture of each genera isolate in same proportion

Efficacy evaluation of biocides on biofilm formation

The efficacy of biocides to control biofilm formation was evaluated by incubating SS chips individually in TSB for pre-decided incubation time (0, 0.5, 1, 1.5 and 2 h). These incubation times are representatives of irreversible stage in biofilm formation. The treatment with three biocides reduced 2-4 \log_{10} CFU/cm² counts of biofilm formed by isolate and their consortia at each incubation time. *Bacillus* cultures showed a significant inhibition when treated with ID and SH in controlling cell counts of the test organism as compared to BKC. The results of present study are in accordance with Ali and Maryam (2012), who reported that BKC was least effective against *Bacillus cereus* because of its ability to form spores. The spores of *B. cereus* have been reported to possess a pronounced ability to adhere to stainless steel, which draws attention in terms of food safety. Results on efficacy of biocide to control biofilms have been presented in Fig.1. These findings are in agreement with results of Pagedar *et al.*, (2010), who also demonstrated

that biofilms were most resistant to sodium hypochlorite and benzalkonium chloride and least to iodophore.

Determination of biofilm formation potential by biomass indicator staining method

The results obtained after analysis indicated that all tested isolates were able to form biofilm in microtitre plate. Our observations in this regard are in agreement with Klausen *et al.*, (2003). The research findings for all the isolates are in agreement with the observations of Pagedar (2007), wherein she studied the development of multiple resistances and their transfer in potential food pathogens of food contact surface biofilms and stated strong biofilm (+++) formation by all selected isolates at 37°C.

Biofilm formation potential on stainless steel chips

All the examined strains expressed the ability of attachment and growth on stainless steel surface when these were incubated in Tryptic

Soy Broth (TSB) at 37°C. The results on biofilm formation potential of isolates on stainless steel chips noted that all selected strains and their consortia possessed stability for biofilm formation on food contact surfaces. Data on biofilm formation of isolates from dairy niche after 48 h incubation, indicated that all strains showed increase of 2-4 log CFU/cm² cell counts as compared to 3 h incubation indicative of final attachment on SS chips was independent of initial attachment. However, marginal difference was recorded in log counts of individual and consortia after 48 h incubation. These results are indicative of possible occurrence of associative growth among different strains.

Determination of inhibitory concentration of biocides against target strains

The results on MIC and MBEC demonstrated that *E. coli* was highly susceptible, whereas, *Salmonella* was highly resistant against iodophore as they showed highest MIC values, while for MBEC; isolates of *Staphylococcus* and *Salmonella* exhibited highest resistance against three biocides in the study and *E. coli* as least resistant. As per MIC, *E. coli* isolates were found highly susceptible, whereas, *Bacillus* and *Pseudomonas* were the most resistant against sodium hypochlorite. As far as MBEC is concerned, *E. coli* species were found to be highly resistant against sodium hypochlorite. The observations on MBEC for benzalkonium chloride demonstrated that, *E. coli* and *Salmonella* were highly susceptible whereas *Bacillus*, *Pseudomonas*, *Listeria*, and consortia, most resistant.

Efficacy evaluation of biocides on biofilm formation

Our *in-vitro* research findings on efficacy evaluation of biocides against biofilms on

food contact surfaces (Stainless Steel chips) showed that sodium hypochlorite and benzalkonium chloride were more effective than iodophore to control preformed biofilms of 0.5 to 1.5 h, however used concentrations of present study were higher than the recommended in-use concentrations. The results revealed that sodium hypochlorite and benzalkonium chloride were more effective in controlling biofilms formed by Gram's negative bacteria on SS chips of attachment time of 1.5 h, as compared to iodophore which was least effective. These can be explained by the fact that penetration of QAC in biofilms was hindered probably due to the interactions of disinfectants with EPS of biofilm matrix which leads to an increased resistance of biofilm (Bridier *et al.*, 2011; Davison *et al.*, 2010). The research observations of the present investigations related to inhibition of biofilm of consortia revealed that sodium hypochlorite and benzalkonium chloride were found most effective as compared to iodophore in controlling biofilms on SS chips. Thus, Application of biocides at used concentrations remained ineffective against biofilms, which will not only compromise food quality and safety but also, would lead in emergence and spread of antimicrobial resistance.

It is possible to prevent the formation of bacterial populations resistant to the disinfection agents by the use of effective cleaning and disinfection procedures and thus prevent the development of hazardous environmental conditions in the food industry and on farms, in many cases. The observations on inhibition of biofilm formation which are at irreversible stage revealed that this can be inhibited by above mentioned biocides' treatment. Biocides used in this study resulted effective reduction in colony counts of minimum 2 log cycles and thus, these concentrations would be able to inhibit biofilm in their initial irreversible

stage in dairy niche. Present study emphasizes the need of a systematic approach for selecting type and concentrations of biocides for application in dairy/food industry to control biofilm at their irreversible stage. The administration of above biocides at their MBEC level would also enable us to control contamination of dairy foods with spoilage and pathogenic microbes due to inhibition of biofilm. Thereby improving food safety and shelf-life of milk and milk products as well as minimise economic losses to dairy industry. Despite the fact, of successful attempts *in vitro* on biofilms, the *in vivo* mechanism is still poorly understood and requires the insights of the molecular mechanism of bacterial biofilms.

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