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Antibiotic Resistance in Co-Culture Biofilm of *Listeria monocytogenes* J0161

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Abstract: Problem statement: Do naturally group-living bacteria express genes the same way as they do in lab grown pure cultures? An intriguing question. *Listeria monocytogenes*, a dreaded pathogen has been and continues to be a subject of study with reference to gene expressions. However, all studies concerning the gene expression of *L. monocytogenes* have been done on pure culture states. Our objective was to study *L. monocytogenes* in a co-cultured state and thereby substantiate that microbes in their natural state of existence are different in their expression than that of the purely cultured lab grown forms. **Approach:** In this study we have focused on the transcriptional and growth response of *L. monocytogenes* to the presence of *Bacillus subtilis* to its niche as planktonic cells and in biofilms. Transcriptional response with reference to Antibiotic Resistance and Synthesis, was studied to elaborate on the differences in gene expression in *L. monocytogenes* as planktonic cells and in biofilm, co-cultured with *B. subtilis*. **Results:** Majority of genes responsible for antibiotic resistance that were up-regulated in co-cultured broth were down regulated in co-cultured biofilm. **Conclusion:** Our observation provides evidence to *L. monocytogenes* being suppressed by *B. subtilis*, however in Biofilms both the species seemed to cooperate with each other towards community living.

Key words: *Bacillus subtilis*, biofilms, co-culture, gene expression, *Listeria monocytogenes*, microarray

INTRODUCTION

Listeria monocytogenes, an opportunistic pathogen is ubiquitously distributed in the natural environment, however due to its importance as a human food-borne and animal pathogen, most ecological studies on the bacterium have focused on food processing and farm environments (Ryser and Marth, 2007). Like in any other environment, *L. monocytogenes* develop growth niches and biofilms within the food processing environments also. The chemical, physical and biotic nature of the microenvironment of biofilms is likely to play an important role in the growth and survival of *L. monocytogenes* within. Biofilm formation by *L. monocytogenes* has been credited to as a major cause for survival and transmission of the pathogen; however, data available on the biology and characteristics of the bacterium, including biofilm formation does not encompass details on bacterium in its original niche. Therefore studies on the ecology of *L. monocytogenes* are necessary and are also pivotal to define the transmission dynamics and control of the bacterium as a pathogen (Ryser and Marth, 2007).

It is known that the observations and data available on the biology and characteristics, of microbes today, have been obtained from lab grown pure cultures However, it is also not veiled that microbes in their natural state of existence are rarely a single species population and therefore it is safe to state that the expressions, be it genotypic or phenotypic is most likely to be different from the data made available to date. So is to state that the microbes that we yearn to eliminate from our surroundings are not single species population either. Does the much hyped discussion on the antibiotic resistance, consider to project on the true natural picture of the phenomenon ? In line with this thought, seconded by few earlier discussions (Ooij, 2011; Foster, 2005; Ryser and Marth, 2007; Nadel *et al*., 2009) we embarked on the pursuit of understanding the expression of *L. monocytogenes* that has been much

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credited for its pertinacity to resist the odds of antimicrobials and antibiotics (Gandhi and Chikindas, 2007) and to survive in its true niche as a denizen of a mixed species population. *L. monocytogenes* has been and continues to be a subject of study with reference to gene expressions (Bowman *et al*., 2008; Chaterjee *et al*., 2006; Liu and Ream, 2008), due to the enormity of risk that it posses. Yet again, all studies concerning the gene expression of *L. monocytogenes* have been done on pure culture states.

Many factors affect the growth of microorganisms in its environment. One of which being interactions with other species in a population. In a co-existing state almost always, every individual microbe is bound to affect the existence of the other, either symbiotically or antagonistically (Alexander, 1974; Nadel *et al*., 2009). Reports on *L. monocytogenes*, in conjunction with the aforesaid statement have already been made. The antagonistic behavior of *Enterococcus durans*, *Lactococcus lactis* and *Lactobacillus plantarum* together to exclude *L. monocytogenes* (Zhoa *et al*., 2004) and the influence of *Staphylococcus aureus* on the population of *L. monocytogenes*, that positively affected the existence of *L. monocytogenes* (Rieu *et al*., 2008) have been reported lately. Despite such reports, the molecular mechanism behind the conflict or co-operation within a microbial community in general had not been taken to research till recently (Garbeva *et al*., 2011).

Bacillus subtilis, a potent producer of antibiotics has been tested and proven to competitively exclude *Salmonella* and *Clostridium Sps* (Ragione and Woodward, 2003). Lately, Torodova and Kozhuharova (2010) established the antimicrobial activity of *B. subtilis* against a range of bacteria, fungi and yeasts. Such reports have been made in the past on inter species interaction and its effects on the potential antibiosis by *B. subtilis* against a wide range of microbes, yet no reports on *B. subtilis* against the dreaded *L. monocytogenes*. For this very reason we opted to study *L. monocytogenes*, as a member of mixed community with *B. subtilis.*

In interaction studies and otherwise, Antibiotic Resistance among microbes, especially the pathogenic ones have been in the seat of discussions for a while now. Understanding the emergence of antibiotic resistance and its implications with reference to clinical and health-care, has been the objective overall. Though our study is on antibiotic resistance we broadened our motive also to understand how a pathogen, in particular *L. monocytogenes*, responds in its expression of antibiosis, to the presence of a probiotic, in this case *B. subtilis*, in its vicinity.

We cultured *L. monocytogenes* as a lone population in broth, while we cultured *L. monocytogenes* also along with *B. subtilis* in broth. Simultaneously *L. monocytogenes* was also co-cultured to form biofilms with *B. subtilis*. We did a gene expression study by microarrays and identified the genes for antibiotics synthesis and resistance in *L. monocytogenes*. The expression pattern of these genes at different intervals in co-culture was demarked.

MATERIALS AND METHODS

Bacterial strains and culture conditions: *L. monocytogenes* J0161 was used in this study, since its complete transcriptome was available and annotated at the *L. monocytogenes* database of the Broads Institute. Also the strain had the highest annotation with the least number of hypothetical proteins listed as per the database. The *L. monocytogenes* J0161 strain was obtained from the Agriculture Research Services (ARS), United States Department of Agriculture (USDA). *B. subtilis* ATCC 11774 was used in the co-culture experiments.

Study on interaction for antibiosis: Overnight cultures grown at 37°C in Tryptone Soy Broth (TSB; catalog no. M011; HIMEDIA) were used for further procedures.

Assay on the competitive exclusion of *L. monocytogenes* by *B. subtilis* in a co-cultured broth: Strain of *B. subtilis* was tested for exclusion of *L. monocytogenes* in a co-cultured broth of TSB. To 8 mL of TSB broth 1 mL of *L. monocytogenes* with a concentration of 7.9 $\log_{10}$ Colony Forming Units (CFU)/ml was inoculated along with 1 mL of *B. subtilis* of concentration 7.0 $\log_{10}$ CFU/mL. 03 such sets of tubes were incubated at 37°C. Also 03 sets of 9 mL TSB with 1 mL of known concentration of *L. monocytogenes* and 9 mL of TSB with 1 mL of known concentration of *B. subtilis* were incubated at 37°C. The sets of tubes as pure cultures were used as controls. At fixed intervals of 24, 48 and 72 h by standard plating technique the cell concentration of *L. monocytogenes* both in co-cultured and pure culture broths were estimated along with the cell concentration of *B. subtilis* in pure culture broth.

Preparation of stainless steel coupons: Stainless steel coupons (AIS 3014) of size the 25×25×1 mm were used for the study. The coupons were detergent washed and dried and a portion of the surface was marked for biofilm growth. An area of 1.5 cm in diameter was encircled. The coupons were sterilized by autoclaving.

Assay on the competitive exclusion of *L. monocytogenes* by *B. subtilis* in a co-cultured biofilm: Biofilms were grown with minor modifications to the protocols described by Zhoa *et al*. (2004). An inoculum of 200 µL of the concentration 2.5 $\log_{10}$CFU/ml of *L. monocytogenes* was deposited on a set of 3 coupons (2×3 Numbers) within the marked area (refer to Section 2.3) of the stainless steel coupons. To one set of the coupons (3 Numbers) pre-deposited with *L. monocytogenes*, 200 µL of the concentration 3.2 $\log_{10}$ CFU/mL of *B. subtilis* were deposited within the marked area. Both the sets of (2 x3) coupons were incubated at 37°C, under humid conditions. Non-adherent bacteria were removed by vacuum aspiration intermittently after every 8 h and replaced with 200 µL of fresh TSB medium. The stainless steel coupons were re-incubated at the same temperature and condition. At each sampling time (24, 48 and 72 h), selected coupons in duplicate were transferred to a laminar flow hood in which the liquid medium from the marked area was removed by vacuum aspiration followed by washing the marked area of each coupon three times with Phosphate Buffer Saline (PBS), to remove the weakly adherent bacteria.

Estimating the inhibition of *L. monocytogenes* by *B. subtilis*: Each of the coupons after the stipulated incubation period were placed in a 50 mL centrifuge tube containing 10 mL of PBS and glass beads (5-mm diameter). The tubes were vortexed for 2 min to disrupt bacteria in the adherent biofilm. The suspended bacteria were serially diluted in PBS and plated in duplicate on TSA for *B. subtilis* count and on Hichrome Listeria agar (catalog no.M1417; Himedia) for *L. monocytogenes* counts. The plates were incubated for 48 h at 37°C and *B. subtilis* counts and *L. monocytogenes* counts were determined. Coupons inoculated with only *L. monocytogenes* served as positive controls, whereas coupons inoculated with only *B. subtilis* served as negative controls. All experiments were duplicated and results are presented as the means.

Microarray studies: Our culture of *L. monocytogenes* J0161, was grown both in broth and as biofilms. The medium used for culturing was TSB. Further, broth cultures were distributed into 02 sets. The first set was to grow *L. monocytogenes* for 24 h at 37°C and the second set was to grow the bacterium in the presence of *B. subtilis* for 4, 12 and 24 h in different tubes individually. Similarly, cultures as biofilms were distributed into 02 sets. The first set was to grow biofilms as pure culture in three different slides, for studying the gene expression after time periods of incubation, from 4, 12-24 h. The second set was to grow biofilms as co-culture of *L. monocytogenes* and *B. subtilis* for 24 h.

RNA extraction and evaluation: Intermittently after 4, 12 and 24 h of incubation as mixed culture broth, 24 h of pure culture and 24 h of mixed biofilm, cells were pelleted and stored in RNA *Later* (catalog no. 7020; Ambion) The cells were then further processed for RNA extraction using Ribo Pure-Bacteria Kit (catalog no. 1925; Ambion) according to the manufacturer's instructions. The concentration and purity of the RNA extracted were evaluated using Bioanalyzer (Agilent; 2100) and absorbance readings at 260nm and 280nm were performed using the Nanodrop Spectrophotometer (Thermo Scientific; 1000).

Probe and microarray slide design: Probes for hybridization were designed for 2973 unique transcripts identified, annotated and available in the database of the Broad's Institute for *L. monocytogenes j0161*, as 60-mer oligonucleotides. Multiple Probes were designed using e-array web tool (Agilent). Probes were to be designed considering the co-culture condition and hence these probes were BLAST against the database of both *L. monocytogenes J0161* and *B. subtilis*. By doing so we eliminated the possible cross-hybridisable probes with the transcripts within *L. monocytogenes* and also with that of *B. subtilis*. were identified and eliminated. Microarray design and layout was also made using the e-array web tool (Agilent) in the 8 x 15K format. Microarray chips with the custom made design and layout was obtained from Agilent.

RNA labelling, amplification and hybridization: The RNA being bacterial, prior to labelling, was Polyadenylated using the Poly(A) Polymerase Tailing Kit (catalog no. PAP5104H; Epicentre Biotechnologies) and the method as described by the manufacturer. Poly (A)-tails were added to the 3'-end of RNA by using A-plus Poly (A) polymerase tailing kit (Epicentre Biotechnologies). Then the samples were labeled using Agilent Quick Amp Kit PLUS (Part number: 5190-0442). Five hundred nanograms each of the samples were incubated with reverse trancription mix at 42°C and converted to double stranded cDNA primed by oligodT with a T7 polymerase promoter. The cleaned up double stranded cDNA were used as template for aRNA generation. cRNA was generated by *in vitro* transcription and the dye Cy3 CTP(Agilent) was incorporated during this step. The cDNA synthesis and *in vitro* transcription steps were carried out at 40°C.

Hybridization, scanning and data analysis: Post amplification the cRNA was subject to hybridization on the Microarray chip. Hybridization was done using the Gene Expression Hybridization kit (Part Number 5188-5242; Agilent). Hybridization was carried out in Sure hyb Chambers (Agilent) at 65ºC for 16 h. The hybridized slides were washed using Agilent Gene Expression wash buffers (Part No: 5188-5327). The microarray slides were then washed with buffers. The hybridized, washed microarray slides were then scanned on G2505C scanner (Agilent Technologies) and Images were quantified using Feature Extraction Software (Version-10.5.1.1, Agilent). The extracted raw data was analysed and normalized using Gene Spring GX 11 Software. The fold values shown are log base $_2$ normalized values.

Pathway annotations: All the Pathway and Gene ontology function data for available strains of *L. monocytogenes* and the protein sequences for available pathway data were collected from Uniprot. Transcript sequences for *L. monocytogenes* J0161 were BLAST against protein database. All the significant genes showing hits greater than 90% identity were selected for Pathway annotation.

Microarray data accession number: The Microarray data have been deposited and made available at the Gene Expression Omnibus database under the accession number GSE27936 (www.ncbi.nlm.nih.gov/geo).

RESULTS

Effect of interspecies interactions on *L. monocytogenes*:

Interaction in broth: The cell concentrations of *L. monocytogenes* and *B. subtilis* at 0 h of incubation were 7.9 $\log_{10}$ CFU/mL and 7.0 $\log_{10}$ CFU/mL respectively. The growth of *L. monocytogenes* after 24 h of Incubation in the presence of *B. subtilis* was estimated. In comparison to the pure culture growth the concentration of *L. monocytogenes* in co-culture state was less. There was a close to 01 $\log_{10}$ reduction CFU/mL. After 24 h Pure culture concentration was 10.1 $\log_{10}$ CFU/mL and in the Co-cultured broth it was 9.25 $\log_{10}$ CFU/mL. Further the growth rate of *L. monocytogenes* after 48 h reduced both incase of *L. monocytogenes* in pure and co-cultured conditions however in the co-cultured condition *L. monocytogenes* the $\log_{10}$ (CFU/mL) reduction was drastic, from 9.25 at 24 h to 7.7 after 48 h as compared to the pure culture which was 8.3 $\log_{10}$ CFU/mL. The drastic reduction was evident after 72 h of incubation. The concentration of *L. monocytogenes* in a co-cultured broth was down to 6.3 $\log_{10}$ CFU/mL after 72 h. Were as in pure culture condition cells grew to a concentration of 10.1 $\log_{10}$CFU/mL after 24 h and thereafter reduced and stabilized at 8.3 and 8.4 $\log_{10}$CFU/mL after 48 and 72 h respectively (Fig. 1).

Interaction in biofilm: The trend of suppression of *L. monocytogenes* was observed also in a co-cultured biofilm with *B. subtilis*, however interestingly, by time, say after 72 h of incubation *L. monocytogenes* were detectable (3.69 $\log_{10}$ CFU/mL) in the mixed culture biofilms.

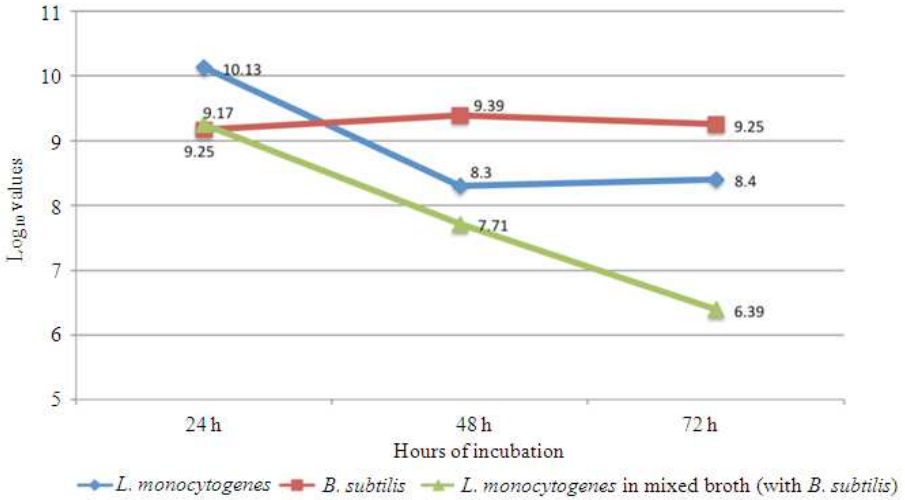

Fig. 1: Growth pattern of *L.monocytogenes* J0161 as pure culture and in the presence of *B.subtilis* in broth, in comparison to growth pattern of B.subtilis pure culture

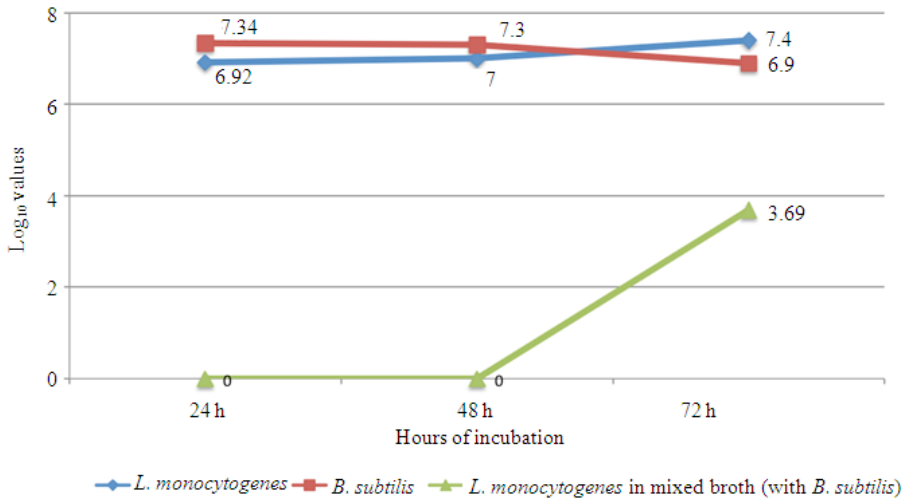

Fig. 2: Growth pattern of *L.monocytogenes* J0161 as pure culture and in the presence of *B.subtilis* in biofilm, in comparison to growth pattern of *B.subtilis* pure culture

L. monocytogenes to an inoculum concentration of 2.5 $\log_{10}$ CFU/mL and *B. subtilis* to a concentration of 3.2 $\log_{10}$ CFU/mL was introduced to the Stainless steel coupons in a volume of 200 µL in the area encircled on the coupons in different sets as described earlier. At 37°C *L. monocytogenes* as pure Biofilms after 24 h of incubation grew to 6.92 $\log_{10}$ CFU cm^{-2}, however *L. monocytogenes* grown as biofilms in the presence of *B. subtilis* after 24 h of incubation were not detectable (<1.7 $\log_{10}$ CFU/cm^2) and after 48 h of incubation *L. monocytogenes* as biofilms in the presence of *B. subtilis* were still undetectable (<1.7 $\log_{10}$ CFU/cm^2), were as *L. monocytogenes* as pure culture after 48 h of incubation had grown to 7.0 $\log_{10}$ CFU/cm^2 concentration. After 72 h of Incubation *L. monocytogenes* was detectable in the biofilms with *B. subtilis*, yet the concentration of the cells was 3.7 $\log_{10}$ less as compared to the concentration seen in *L. monocytogenes* as pure biofilms after 72 h (Fig. 2).

Transcriptional response of *L. monocytogenes* to *B. subtilis*: As compared to 24 h pure culture broth, of 2974 genes transcripts, 469 and 515 genes were positively regulated in *L. monocytogenes* in co-cultured broth and biofilms with *B. subtilis*, respectively, out of which 281 genes were commonly up-regulated in both the conditions. 188 genes specific to co-culture broth and 234 specific to biofilms were up- regulated.

Expression of antibiotic resistance in co-culture broth: By comparing the gene expression of *L. monocytogenes* in 24 h pure broth culture to that of *L. monocytogenes* co-cultured with *B. subtilis*, for 4, 12 and 24 h, we were able to identify a pattern in the expression of antibiotic resistance and biosynthesis by *L. monocytogenes* in response to the presence of *B. subtilis*. Specifically 15 gene transcripts as listed in Table 1, all of them majorly in response to antibiotics were Up-regulated. The pattern was either an ascend from 0.5 to 2.0 fold up-regulation from 4, 12 to 24 h or from Negative regulation to 2.0 fold positive up-regulation.

08 of the 15 genes were identified based on gene annotations as available in the database as described earlier, to be directly responsible for drug resistance, four of which coded for general drug resistance transporters (LMOG_01715, LMOG_02237, LMOG_00072 and LMOG_00745) and one for multidrug resistance transporter (LMOG_00292). 03 other drug resistance transporters were specific to *Fosmycin resistance* (LMOG_02826), *Lincomycin resistance* (LMOG_02995) and *Tetracycline resistance* (LMOG_00897). For other genes since the pathway annotation was not available for *L. monocytogenes* J0161, they were extracted from Uniport as described earlier. From the extracted pathway annotations, 02 genes coding for Penicillin binding proteins (LMOG_00981 and LMOG_ 01349), 02 for major facilitator family transporter (LMOG_00858 & LMOG_01645), 01 for Efflux pump Lde (LMOG_01756) and 02 others for *Nucleotidyltransferase* (LMOG_02571) and *Acyltransferase* (LMOG_02820) were also found to be responsible for antibiotic resistance in the species showing the closest hit on BLAST.

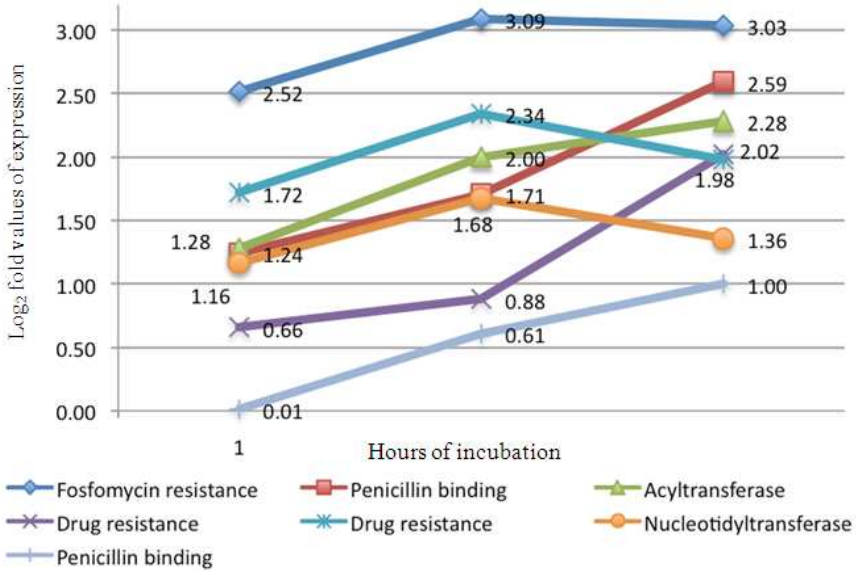

Fig. 3: Antibiotic Resistance genes positively regulated in co-culture broth

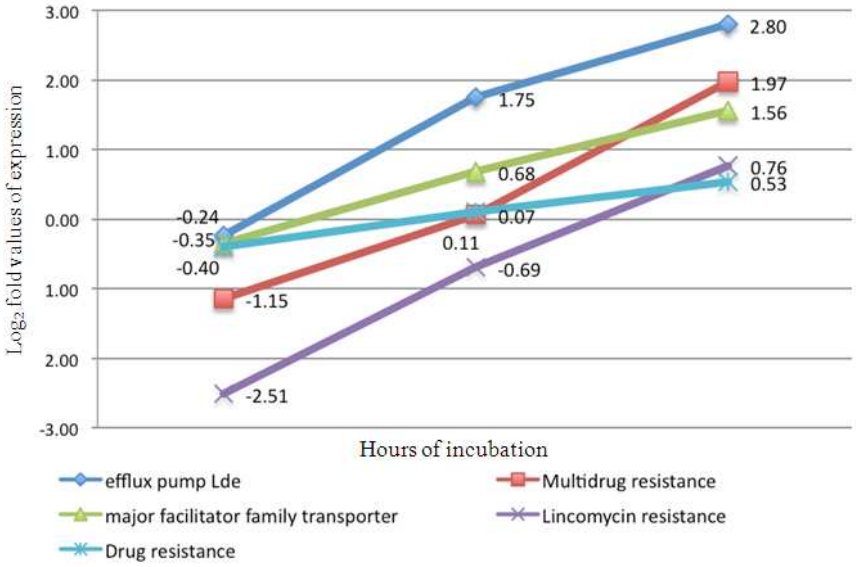

Fig. 4: Antibiotic Resistance genes ascending to positive regulation from negative regulation

Genes positively regulated continually: Among the 15 antibiotic resistance response transcripts, 07 were continuously up-regulated and expressed positive regulation early from the fourth hour of incubation, namely genes for *Fosfomycin resistance* (LMOG_02826T0), general drug resistance (LMOG_00072T0 and LMOG_02237T0), *acyltransferase* (LMOG_02820T0), *penicillin binding* (LMOG_01349T0 & LMOG_00981T0) and *nucloetidyl transferase* (LMOG_02571Y0) (Fig. 3).

Genes ascended to positive regulation from negative regulation: 05 of the other 08 transcripts, namely Efflux pump Lde (LMOG_01756T0), *Multidrug resistance* (LMOG_00292T0), Major facilitator family transporter (LMOG_00858T0), *Lincomycin resistance* (LMOG_02995T0) and general drug resistance (LMOG_00745T0) gradually shifted to be positively regulated from negative regulation (Fig. 4). However , the ascend towards positive regulation was steep in Efflux pump Lde and Major facilitator family transporter.

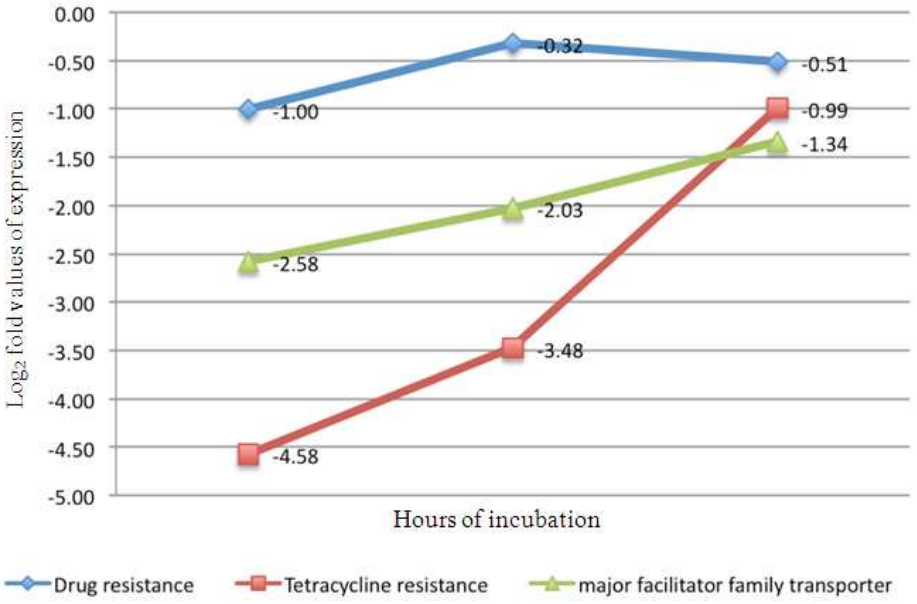

Fig. 5: Antibiotic resistance genes negatively regulated, yet ascending towards positive regulation

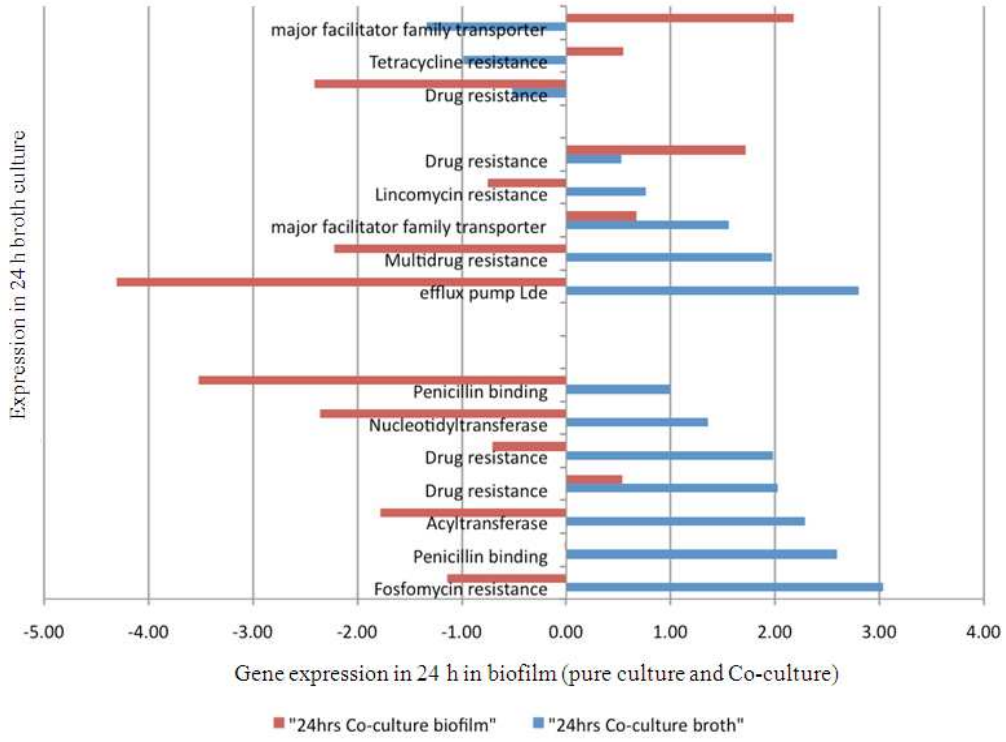

Fig. 6: Expression of antibiotic resistance in co-culture biofilm

Genes negatively regulated, yet ascending towards positive regulation: 03 of the 15 genes showed an ascend, but within the negative fold expression (Fig. 5). Namely, for general drug resistance, Tetracycline resistance and major facilitator family transporter (LMOG_01715T0, LMOG_00897T0 and LMOG_01645T0).

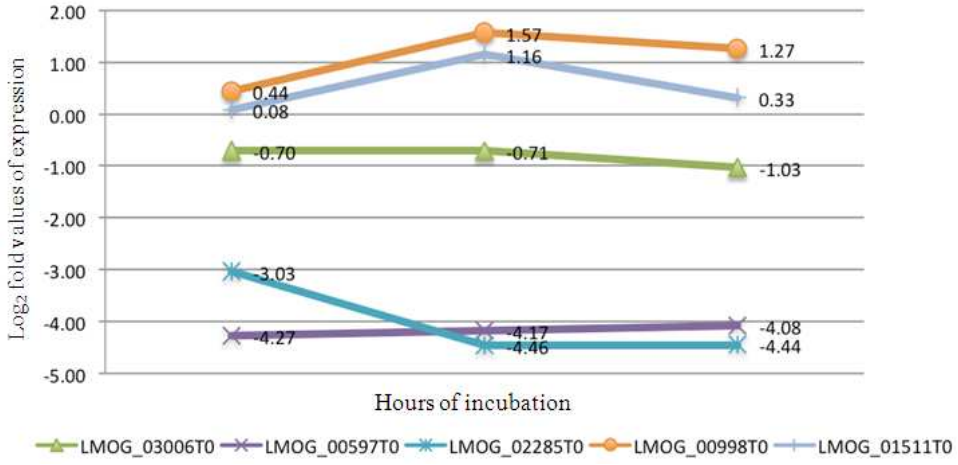

Fig. 7: Expression of Antibiotic Biosynthesis in co-cultured broth state

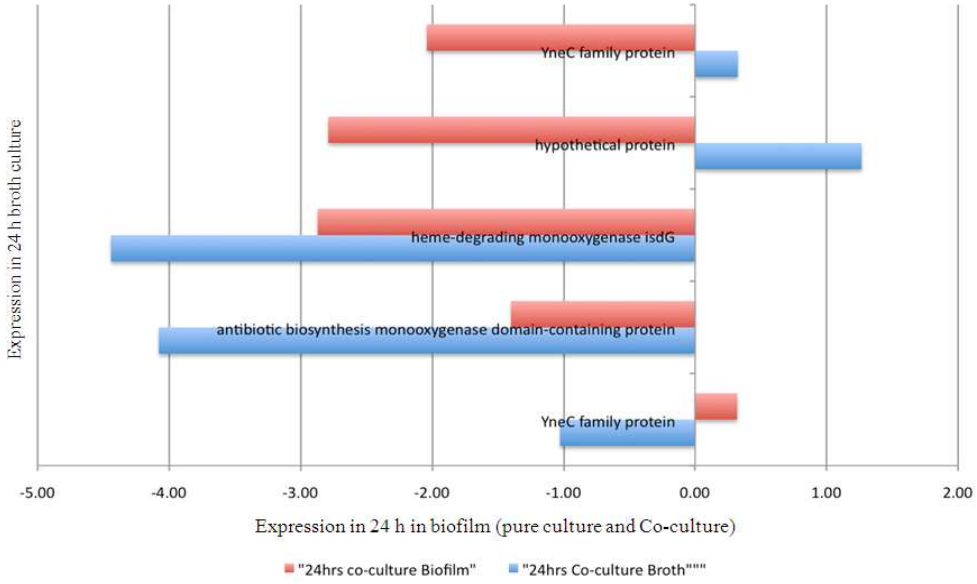

Fig. 8: Expression of antibiotic biosynthesis in co-culture broth and biofilm

Expression of antibiotic resistance in co-culture biofilm: The response of *L. monocytogenes* to the presence of *B. subtilis* in biofilm with reference to expression of antibiotic resistance and biosynthesis was possible by comparing the 24 h Pure broth culture and 24 h Co-culture broth to 24 h Co-cultured biofilm. Contrary to the Up-regulation and or ascending to positive regulation of the 15 antibiotic resistance genes in the co-culture broth, but for 02 genes, a general drug resistance (LMOG_00745T0) and a facilitator family transporter gene (LMOG_01645T0) all other genes (LMOG_01715, LMOG_02237, LMOG_00072, LMOG_00745,LMOG_00292, LMOG_02826, LMOG_02995, LMOG_00897) were either negatively or neutrally regulated in biofilms (Fig. 6).

Expression of antibiotic biosynthesis: Concurrently the expression of genes identified for antibiotic biosynthesis in *L.monocytogens* J0161, namely LMOG_03006T0 (*YneC* family), LMOG_00597T0 (*monooxygenase*), LMOG_02285T0 (*monooxygenase*), LMOG_00998T0 (*hypothetical protein*) and LMOG_01511T0 (*YneC family*) were mostly negatively regulated in co-cultured broth state (Fig. 7). Also in co-cultured biofilm after 24 h of incubation the 05 genes remained negatively or neutrally regulated (Fig. 8).

Table 1: Antibiotic Resistance response genes and their responses

Type of Regulation	S. No	Gene	Gene Description	Log_2 fold expression values 4 h Co-culture broth	12 h Co-culture broth	24 h Co-culture broth	24 h Co-culture biofilm
Continually positively regulated							
	1	LMOG_02826T0	Fosfomycin Resistance	2.52	3.09	3.03	-1.14
	2	LMOG_01349T0	Penicillin binding	1.24	1.71	2.59	-0.01
	3	LMOG_02820T0	Acyltransferase	1.28	2.00	2.28	-1.78
	4	LMOG_00072T0	Drug resistance	0.66	0.88	2.02	0.54
	5	LMOG_02237T0	Drug resistance	1.72	2.34	1.98	-0.71
	6	LMOG_02571T0	Nucleotidyltransferase	1.16	1.68	1.36	-2.36
	7	LMOG_00981T0	Penicillin binding	0.01	0.61	1.00	-3.52
Ascending from negative to positive regulation							
	1	LMOG_01756T0	Efflux pump Lde	-0.24	1.75	2.80	-4.31
	2	LMOG_00292T0	Multidrug resistance	-1.15	0.07	1.97	-2.22
	3	LMOG_00858T0	Major facilitator family transporter	-0.35	0.68	1.56	0.67
	4	LMOG_02995T0	Lincomycin resistance	-2.51	-0.69	0.76	-0.75
	5	LMOG_00745T0	Drug resistance	-0.40	0.11	0.53	1.72
Negatively ascending regulation							
	1	LMOG_01715T0	Drug resistance	-1.00	-0.32	-0.51	-2.41
	2	LMOG_00897T0	Tetracycline resistance	-4.58	-3.48	-0.99	0.55
	3	LMOG_01645T0	Major facilitator family transporter	-2.58	-2.03	-1.34	2.18

DISCUSSION

The objective of our study was to understand the basic population behavior in terms of the growth pattern of *L. monocytogenes* to the presence of *B. subtilis* in its habitat. Further, we wanted to observe the gene expression pattern of *L. monocytogenes* in response to the presence of *B. subtilis*, both in broth and biofilms.

The novelty of our study was that, we have considered to study the gene expression profile of a pathogen in a co-existing state. Due to problems related to cross-hybridization of RNA transcripts of the non-target organisms with the probes designed for the transcripts of the target organisms, such experiments could have not been designed. However, in our experiments, we designed probes specific to transcripts of *L. monocytogenes* J0161, with adequate measures to eliminate cross hybridization with transcripts of *B. subtilis.* Though the whole gene expression profile of *L. monocytogenes* has been extracted, in this manuscript we have reserved our discussions to expression of antibiotic resistance and synthesis related genes only.

We observed that, as planktonic cells in broth, *B. subtilis* suppressed *L. monocytogenes.* The population of *L. monocytogenes* reduced by time. However within a biofilm, *L. monocytogenes* seemed to thrive and both *B. subtilis* and *L. monocytogenes* could be enumerated, exhibiting pertinacity to life. They seem to co-exist. In fact, the number of *L. monocytogenes* cells seemed to increase in number gradually within a biofilm with *B. subtilis*. This behavior, was a finding not reported so far, hence we studied further on this behavioral pattern to the molecular level.

We designed an experimental setup to bring the bacterial cells together in both the states of growth, broth and biofilm as co-cultures and studied the transcriptional expression of *L. monocytogenes*. The expression as we pre-empted were different in both the states of growth, in particular after 24 h of culturing. Intermittent extraction of RNA (4 and 12 h) from the co-culture broth gave us a time mapped expression pattern.

L. monocytogenes all through the times in the culture state from 4-24 h both in broth and in biofilms did not respond offensively. Of the annotated genes, related to antibiotic synthesis, none were Up-regulated. Infact, as compared to the 24 h mono broth culture of *L. monocytogenes*, but for two genes, the antibiotic synthesis gene expression were being negatively regulated. Even the two genes up-regulated gradually seemed to dip in the fold expression after 12 h.

In contrast to the antibiotic synthesis, expression of antibiotic resistance related genes were up-regulated in broth and down-regulated in biofilms. We considered expression of antibiotic resistance related genes, as an indirect indicator to antibiosis by *B. subtilis*, by comparing the gene expression data to our corresponding results from the culture inhibition experiments.

Comparing the gene expression observation to the growth pattern of *L. monocytogenes* in broth, suggests that the suppression of *L. monocytogenes* by *B. subtilis* is attributed to the antibiosis expressed by *B. subtilis.* Further, in deviation to what was observed as positively regulated expression in co-cultured broth, antibiotic resistance related genes were negatively regulated in

co-culture biofilm. Only 02 of the 15 antibiotic resistance genes were being positively regulated. Comparing this gene expression observation to the growth pattern of *L. monocytogenes* in biofilm with *B. subtilis*, we could say that *B. subtilis* did not shown antibiosis to *L. monocytogenes* in the biofilms, which in turn could mean that within a biofilm both *L. monocytogenes* and *B. subtilis* might cooperate to co-exist. The gene expression pattern by *L. monocytogenes* in co-culture broth and biofilm with reference to genes on antibiotic response and synthesis corresponded to our observation on the growth pattern.

Variations in the expression of antibiotic resistance from one state of growth to the other may be attributed and specific to the conditions defined in our study, yet it is noteworthy to mention that due to high degree of uncertainty in studies involving biological systems, it is crucial that a phenomenon graded critical, such as the antibiotic resistance, be observed in the most plausible conditions of existence. Much has been accounted to on the resistance to antibiotics, but all that is available, detail out on the expression as if the microbes existed as a pure culture population.

CONCLUSION

We could conclude that two different bacterial species that compete in broth are actually co-existing in a biofilm. We infer that the behavior of a microbe may change from one state of growth to other and further more in the presence of another bacterium. Further, it will be appropriate for us to recommend that studies involving not only antibiotic resistance or synthesis but also in general on the metabolic patterns and expressions of microbes be done bearing in mind the fact that they seldom exist in pure (mono) culture state in their natural environment.

Ryser and Marth (2007), in summing up the future research perspective on *Listeria*, had prophesied that characterization of microbial communities in association with *Listeria* would be the focus of research, with hopefully interesting revelations. Our findings, we believe, substantiate and also provide ample scope for studies in this direction.

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REFERENCES

Alexander, R.D., 1974. The evolution of social behavior. Annu. Rev. Ecol. Syst., 5: 325-383. DOI: 10.1146/annurev.es.05.110174.001545

Bowman, J.P., C.R. Bittencourt and T. Ross, 2008. Differential gene expression of *Listeria monocytogenes* during high hydrostatic pressure processing. Microbiology, 154: 462-475. DOI: 10.1099/mic.0.2007/010314-0

Chaterjee, S.S., H. Hossain, S. Otten, C. Kuenne and K. Kuchmina *et al*., 2006. Intracellular gene expression profile of *Listeria monocytogenes*. Infect. Immun., 74: 1323-1338. DOI: 10.1128/ IAI.74.2.1323-1338.2006

Foster, K.R., 2005. Biomedicine. Hamiltonian medicine: Why the social lives of pathogens matter. Science, 308: 1269-1270. PMID: 15919984

Gandhi, M. and M.L. Chikindas, 2007. Listeria: A foodborne pathogen that knows how to survive. Int. J. Food Microbiol., 113: 1-15. PMID: 17010463

Garbeva, P., M.W. Silby, J.M. Raaijmakers, S.B. Levy and W. Boer, 2011. Transcriptional and antagonistic responses of *Pseudomonas fluorescens* Pf0-1 to phylogenetically different bacterial competitors. ISME J., 5: 973-985. PMID: 21228890

Liu, Y. and A. Ream, 2008. Gene expression profiling of *Listeria monocytogenes* strain F2365 during growth in ultrahigh-temperature-processed skim milk. Applied Environ. Microbiol., 74: 6859-6866. DOI: 10.1128/AEM.00356-08

Nadel, C.D., J.B. Xavier and R.K. Foster, 2009. The sociobiology of biofilms. FEMS Microbiol. Rev., 33: 206-224. PMID: 19067751

Ooij, C.V., 2011. Microbial ecology: Strong fences make good neighbours., Nat. Rev. Microbiol., 9: 150-151. PMID: 21451564

Ragione, R.M.L. and M.J. Woodward, 2003. Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype *Enteritidis* and *Clostridium perfringens* in young chickens. Vet. Microbiol., 94: 245-256. PMID: 12814892

Rieu, A., J.P. Lemaitre, J. Guzzo and P. Piveteau, 2008. Interactions in dual species biofilms between *Listeria monocytogenes* EGD-e and several strains of *Staphylococcus aureus*., Int. J. Food Microbiol., 126: 76-82. PMID: 18554739

Ryser, E.T. and E.H. Marth, 2007. Listeria, Listeriosis and Food Safety. 3rd Edn., CRC Press, Inc. New York, ISBN: 0824757505, pp: 873.

Torodova, S. and L. Kozhuharova, 2010. Characteristics and antimicrobial activity of *Bacillus subtilis* strains isolated from soil. World J. Microbiol. Biotechnol., 26: 1207-1216. DOI: 10.1007/s11274-009-0290-1

Zhoa, T., M.P. Doyle and P. Zhoa, 2004. Control of *Listeria monocytogenes* in a biofilm by competitive-exclusion microorganisms., Applied Environ. Microbiol., 70: 3996-4003. DOI: 10.1128/AEM.70.7.3996-4003.2004