

Loss of crystal violet binding activity in stationary phase *Yersinia enterocolitica* following gamma irradiation

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Ionizing radiation can eliminate virulent Yersinia enterocolitica from meat. It is possible, however unlikely, that a small number of Y. enterocolitica could survive the pasteurization process. The virulence of Y. enterocolitica is dependent upon the presence of a 70 kb plasmid. The effect of low-dose ionizing radiation on the plasmid-associated virulence trait of crystal violet binding was investigated. Y. enterocolitica strains which carried the virulence plasmid were suspended in Butterfield's Phosphate Buffer or raw ground pork and irradiated to a dose of 1.0 or 1.25 kGy, respectively. Loss of crystal violet binding increased 10-fold following exposure to ionizing radiation, regardless of the suspending medium. Polymerase chain reaction analysis of Y. enterocolitica isolates that did not bind crystal violet following irradiation indicated that loss of the virulence plasmid, as opposed to mutation of a single plasmid-encoded gene, was the major mechanism for elimination of the crystal violet binding trait.

Introduction

Yersinia enterocolitica causes foodborne illness in humans (Doyle and Cliver 1990, Kapperud 1991). Yersiniosis affects an estimated 96 000 individuals annually in the United States. Ninety percent of those cases are the result of foodborne transmission (Mead et al. 1999). *Yersinia enterocolitica* can be isolated from beef, poultry, lamb and ready-to-eat meat products (Hanna et al. 1976, Myers et al. 1982, Brocklehurst et al. 1983, Schiemann 1987, Mattila and Frost 1988,

Kwaga and Olson 1991, Toora et al. 1994). It is considered the most important bacterial foodborne pathogen by the pork production and processing industry in the United States (Davies 1997). Like *Listeria monocytogenes*, *Y. enterocolitica* is capable of growth at refrigeration temperatures and in high salt environments (Sutherland and Bayliss 1994). Because of those characteristics, *Y. enterocolitica* is of particular concern as a foodborne pathogen.

Ionizing radiation can eliminate *Y. enterocolitica* from meat products (Tarkowski et al. 1984, Grant and Patterson 1991, Kamat et al. 1997, Shenoy et al. 1998). However, because exposure to low-dose ionizing radiation is a pasteurization as opposed to a sterilization process, it is possible that a small number of pathogenic bacteria could survive the treatment. While it is theoretically possible that exposure to ionizing

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radiation could produce a virulent organism from a non-virulent parent, no such incident has been documented (Anonymous 1994). Conversely, treatment of food-borne pathogens with ionizing radiation leads to a loss of virulence (Ingram and Farkas 1977, Anonymous 1994; Naidu et al. 1998). The mechanisms by which foodborne pathogens are attenuated by ionizing radiation have not been extensively studied.

Exposure to ultraviolet radiation, antibiotics that inhibit the enzyme DNA gyrase, or exposure to genotoxic chemicals induce the loss of plasmids in bacteria and fungi (Danilevskaya and Gregarov 1980, Cejka et al. 1982, Cansado et al. 1989, Nakamura 1990). Many foodborne pathogens carry plasmids that contribute to their virulence, including *Escherichia coli*, *Salmonella* spp., and *Shigella* spp. (Sansone et al. 1983, Kawahara et al. 1988, Barrow and Lovell 1988, Sasakawa et al. 1992). Virulence of *Yersinia* spp. is dependent on the presence of structurally related plasmids (Cornelis et al. 1998). Loss of the virulence plasmid, or mutation of plasmid encoded virulence genes, leads to attenuation of *Y. enterocolitica* strains (Darwin and Miller 1999). Because of the extensive structural characterization of the *Y. enterocolitica* virulence plasmid, and the phenotypes associated with it, it is a model system in which to study ionizing radiation-induced attenuation of foodborne pathogens. Plasmid loss may represent one mechanism by which foodborne pathogens are attenuated following irradiation.

In this work, the effect of ionizing radiation on the maintenance of *Y. enterocolitica*'s plasmid-linked virulence trait of crystal violet (CV) binding (Bhaduri et al. 1987) was investigated. The following questions were addressed: (1) to what extent did exposure to ionizing radiation affect the virulence plasmid associated trait of CV binding? (2) was the loss of the CV binding trait due to mutation of a plasmid-encoded gene or loss of the large virulence plasmid? (3) was there a difference in the radiation resistance of the two *Y. enterocolitica* strains used? (4) could the results obtained using an *in vitro* buffer system be translated to *Y. enterocolitica* suspended in a meat product?

Materials and Methods

Strains

Two virulence plasmid-bearing strains of *Y. enterocolitica* (YEP⁺) were utilized. YEP⁺ strain 51871 (serotype O:8) was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). Strain GER P⁺ (serotype O:3) was obtained from S. W. Weagant (U. S. Food and Drug Administration, Bothell, WA, USA). The strains were propagated on brain-heart infusion agar (BHIA) (Difco Laboratories, Detroit, Mississippi, USA) at 27°C and maintained at 2°C until ready for use. Strain identity was confirmed with gram-negative identification (GNI) cards using the Vitek Automicrobic System (bioMerieux Vitek, Inc., Hazelwood, Missouri, USA).

Presence or absence of the virulence plasmid was verified by CV binding assay, plasmid DNA isolation, and polymerase chain reaction (see below). The YEP⁺ strains were routinely re-isolated and tested for the crystal violet binding phenotype to maintain the spontaneous CV binding loss rate below 1%. The plasmidless derivatives of each strain, 51872 (ATCC, Manassas, VA, USA) and GER P⁻ (Bhaduri et al. 1988), were used as controls throughout the course of the study.

Stationary phase YEP⁺ cultures were used for determination of D_γ. Stationary phase bacterial cells, as opposed to actively growing bacteria, are more resistant to the effects of ionizing radiation (Kelner et al. 1955, Thayer and Boyd 1993). Therefore, both the radiation-resistance and loss of CV were quantified using YEP⁺ in their most radiation-resistant state.

Meat

Centre-cut pork chops were purchased from a local market and ground to approximately 3.2 mm diameter. The ground pork was then aliquoted (100 g) into No. 400 Stomacher bags (Tekmar, Inc., Cincinnati, Ohio, USA) and vacuum packed to 0.23 mmHg using a Multi-Vac A300 Vacuum-Packager (Kansas City, Missouri, USA). In order to eliminate contaminating micro-organisms, the meat was then sterilized using the protocol of Thayer et al.

(1995) by irradiating to a dose of 42 kGy (-30°C). The meat was then stored at -70°C until ready for use.

Gamma irradiation

A Lockheed Georgia Company self-contained ^{137}Cs irradiator was used for all exposures. The radiation source consisted of 23 individually sealed source pencils placed in an annular array. The 22.9×63.5 cm cylindrical sample chamber was located central to the array when placed in the operating position. *Yersinia enterocolitica*-inoculated samples were placed vertically in the sample chamber to insure uniformity of dose. The dose rate was $0.103 \text{ kGy min}^{-1}$. The temperature during irradiation was maintained at $4 \pm 1.0^{\circ}\text{C}$ by the gas phase from a liquid nitrogen source, which was introduced directly into the top of the sample chamber. The temperature was monitored using two thermocouples placed in close proximity to the samples. The dose delivered was verified using 5 mm alanine pellet dosimeters, which were then measured using a Bruker EMS 104 EPR Analyzer.

D_{γ} is the gamma radiation dose required to reduce the number of viable micro-organisms by 90%. The radiation doses used for D_{γ} determination were 0.2, 0.4, 0.6, 0.8 and 1.0 kGy for YEP⁺ suspended in BPB and 0.25, 0.5, 0.75, 1.25 kGy when raw ground pork was the suspending medium. Higher radiation doses were used for D_{γ} determination in raw ground pork due to the higher radiation resistance of micro-organisms when suspended in meat as opposed to buffer. The non-irradiated control values were not used for determination of D_{γ} .

D_{γ}

The YEP⁺ strains were cultured independently in 100 ml brain-heart infusion medium (Difco Laboratories) in baffled 500 ml Erlenmeyer culture flasks at 27°C (150 rpm) for 18 h. The bacteria were then sedimented by centrifugation and resuspended in a 10-fold reduced volume of Butterfield's Phosphate Buffer (BPB) obtained from Applied Research Institute (Newtown, Connecticut, USA). For determination of D_{γ} in BPB, the resuspended YEP⁺ strains were

aliquoted (5 ml) into sterile borosilicate glass tubes and maintained on ice until ready for irradiation. When ground pork was the suspending medium, the concentrate was diluted 1/10 into 100 g sterile ground pork and mixed for 90 s in a Stomacher Mixer (Tekmar, Inc., Cincinnati, Ohio, USA). The inoculated meat was then aliquoted (5 g) into No. 400 Stomacher bags, vacuum-packaged, and refrigerated until ready for irradiation. Following irradiation, the meat samples were diluted 1/10 in sterile BPB and mixed by stomaching for 90 s. The resuspended YEP⁺ strains were then serially diluted in sterile BPB and surface plated to BHIA. The plates were incubated approximately 24 h at 37°C and the number of colony forming units (cfu) per plate determined. Following enumeration of cfu/plate, the rate of CV binding was also determined. The average (N) cfu/plate of the irradiated samples at each dose were divided by the average cfu/plate of the zero dose (N_0) to produce a survivor ratio (N/N_0). Each experiment was conducted independently three times.

Crystal violet binding assay

The procedure outlined by Bhaduri et al. (1987) was used. Only colonies that clearly failed to bind the dye were scored as CV⁻ clones, leading to a possible under-estimation of the phenotype loss rate. *Y. enterocolitica* CV⁻ colonies obtained following the 1.0 kGy dose were randomly selected, re-isolated, and re-tested for the phenotype.

PCR amplification of plasmid encoded and chromosomal DNA

Multiplex PCR using DNA primers to the virulence plasmid encoded regulatory gene *virF* and the chromosome encoded *ail* gene (Bhaduri et al. 1998) was used to verify the presence of plasmid encoded DNA sequences and confirm *Y. enterocolitica* identity. Following PCR, the amplified DNA fragments were separated via agarose gel electrophoresis, visualized with ethidium bromide and UV transillumination at 302 nm (Bhaduri et al. 1980) and photographed for maintenance as a permanent

record. Amplified DNA fragments and requisite controls are shown in Fig. 1.

Statistical analysis

Statistical analysis was completed using SAS/STAT Version 6.12 (SAS Institute, Inc., Cary North Carolina, USA) and Sigma Plot Version 5.0 (SPSS, Inc., Chicago, Illinois, USA). Population reduction data were analysed by analysis of variance using the general linear model procedure of the SAS statistical package (Freund et al. 1986, SAS Institute 1987). Comparison of regressions was performed by analysis of covariance (Thayer et al. 1995).

Results

Survival curves for strains ATCC 51871 and GERP⁺ suspended in BPB are shown in Fig. 2. The D_{γ} obtained for strain ATCC 51871 was 0.17 ± 0.09 kGy (Fig. 2(a)) as opposed to 0.19 ± 0.05 kGy for strain GERP⁺ (Fig. 2(b)). Analysis of covariance ($n=3$, $\alpha=0.05$) indicated no difference in D_{γ} between the two YEP⁺ strains when suspended in BPB. The D_{γ} obtained for ATCC 51871 was 0.21 ± 0.07 kGy (Fig. 3(a)), as opposed to 0.19 ± 0.07 kGy for GERP⁺ (Fig. 3(b)), when the strains were suspended in raw ground pork (RGP). Analysis of covariance ($n=3$, $\alpha=0.05$) indicated no differ-

ence in D_{γ} between the two YEP⁺ strains when suspended in RGP.

The percentage of YEP⁺ colonies that failed to bind CV following irradiation in BPB is shown in Fig. 4. The rate of CV⁻ colonies for the untreated controls was $0.27 \pm 0.02\%$ for ATCC 51871 (Fig. 4(a)). The CV⁻ rate increased with dose to $5.62 \pm 0.99\%$ at the 1.0 kGy dose. The increase became statistically significant at the 0.6 kGy dose as determined by Student's t test ($n=3$, $\alpha=0.05$). The rate of CV⁻ colonies for GERP⁺ (Fig. 4(a)) was $0.32 \pm 0.04\%$ in the untreated controls versus $3.38 \pm 0.77\%$ at the 1.0 kGy dose. The increase became statistically significant at a dose of 0.4 kGy ($n=3$, $\alpha=0.05$). The combined (pooled) data set for both strains is also shown in Fig. 4(a). The increase in CV⁻ became significant at 0.4 kGy for the combined data set.

The percentage of YEP⁺ colonies that failed to bind CV following irradiation in RGP is shown in Fig. 4(b). The rate of CV⁻ colonies for the untreated controls was $0.53 \pm 0.17\%$ for ATCC 51871 and increased with dose starting at 0.5 kGy (Fig. 4(b)). The increase became statistically significant at the 0.5 kGy dose as determined by Student's t -test. The rate of CV⁻ colonies for GERP⁺ was $0.46 \pm 0.26\%$ in the untreated controls versus $0.82 \pm 0.63\%$ at the 1.25 kGy dose (Fig. 4(b)). The increase became statistically significant at a dose of 0.5 kGy. Pooled data is shown in Fig. 4(b). The increase in CV⁻ became significant at 0.5 kGy. Analysis

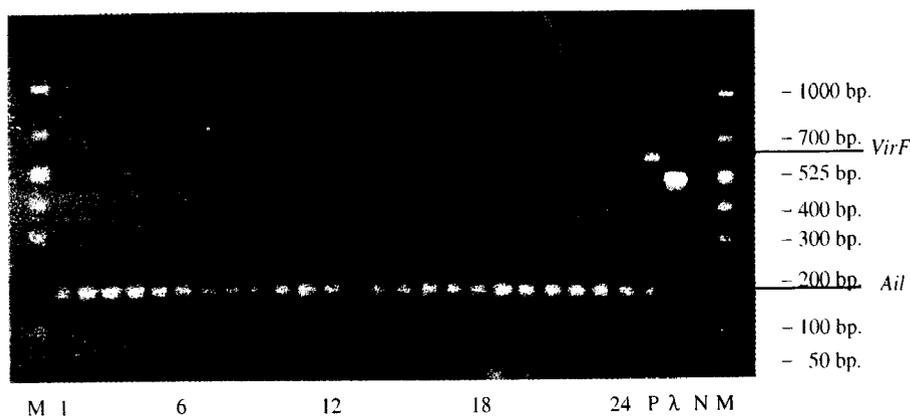
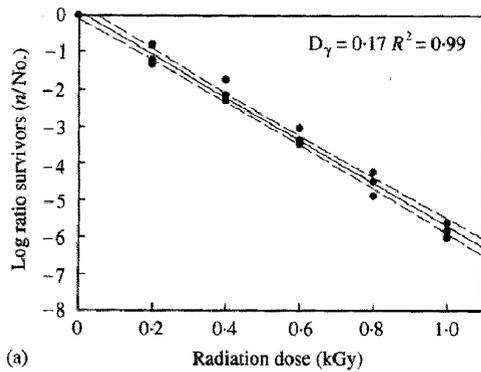
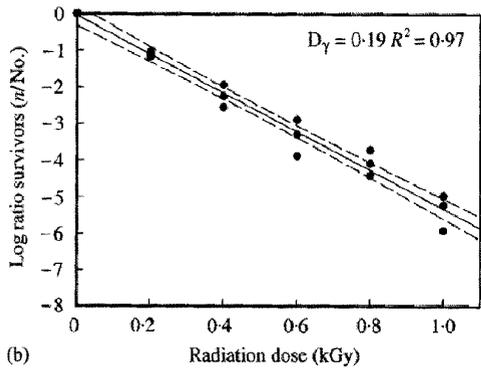


Figure 1. Multiplex PCR analysis of CV⁻ clones. Margins, 50–1000 bp ladder marker. Lanes 1–24, CV⁻ clones showing the loss or presence of 591bp (*virF*) of 170 bp (*ail*) PCR products. Lane P, YEP⁺ strain showing the amplification of 170bp (*ail*) and 591 (*virF*) PCR products. Lane λ , Phage λ control for the PCR assay. Lane N, PCR reaction mix and primer control with no template.

Radiation-induced plasmid loss in *Y. enterocolitica*

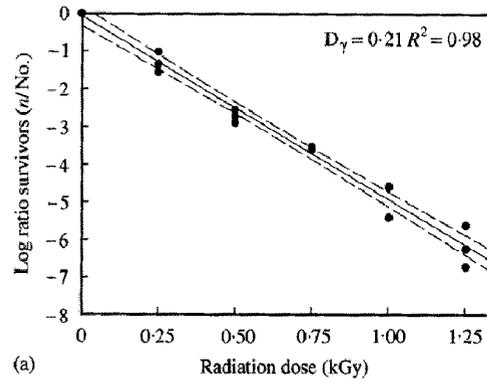


(a)

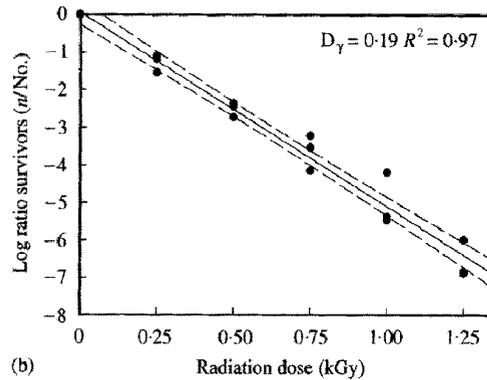


(b)

Figure 2. Radiation Resistance of *Y. enterocolitica* in BPB. Pooled regression curve (solid line) with 95% confidence intervals (dashed lines) for strain ATCC 51871 (a) and strain GERP⁺ (b).



(a)



(b)

Figure 3. Radiation Resistance of *Y. enterocolitica* in pork. Pooled regression curve (solid line) with 95% confidence intervals (dashed lines) for strain ATCC 51871 (a) and strain GERP⁺ (b).

of covariance ($n=3$, $\alpha=0.05$) indicated no difference in the dose dependent rate of CV loss between the two YEP⁺ strains regardless of the suspending medium.

Y. enterocolitica colonies that did not bind CV following a radiation exposure of 1.0 kGy, from both pork and BPB, were isolated and tested for presence of the virulence plasmid. Attempts to visualize purified plasmid DNA following agarose gel electrophoresis yielded negative results (data not shown). PCR results from samples of 24 CV⁻ clones are shown in Fig. 1 (Lanes 1–24). The primer pair for the plasmid-encoded *virF* gene failed to amplify the 591 bp *virF* DNA product in each of the 24 CV⁻ clones tested. The 591 bp *virF* DNA was amplified in the positive control (GERP⁺) strain (Fig. 1, Lane P). However, each plasmidless CV⁻ isolate, including the positive control (GERP⁺) strain, amplified a 170 bp product to

confirm to the presence of the chromosomal *ail* gene, identifying them as pathogenic *Y. enterocolitica* (Fig. 1, Lanes 1–24, P). This indicated that plasmid loss, as opposed to mutation of a single plasmid encoded gene responsible for the CV phenotype, was the primary mechanism for the loss of CV activity in the *Y. enterocolitica* irradiated to a dose of 1.0 kGy.

Discussion

Radiation induced loss of CV binding activity increased, in a dose-dependent manner, in both of the YEP⁺ strains tested regardless of the suspending medium. The major mechanism by which CV binding was lost, at a radiation dose required to reduce the viable population by five log (10) in number, was through loss of the virulence plasmid. The D_γ for the YEP⁺ strains, in

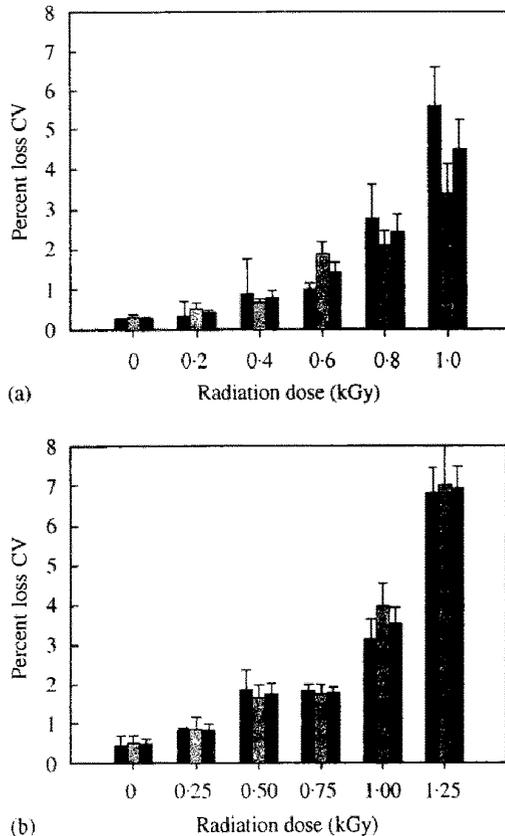


Figure 4. Rates of ionizing radiation-induced CV. Rate of CV⁻ in *Y. enterocolitica* ATCC 51871 (■) and GERP⁺ (▨) and combined ATCC 51871 and GERP⁺ (■) following treatment with ionizing radiation when suspended in BPB (A) or raw ground pork (B). Standard error bars ($n = 3$) are included on each column.

both liquid and pork suspensions, were consistent with those obtained in other studies (Tarkowski et al. 1984, Grant and Patterson 1991, Kamat et al. 1997, Shenoy et al. 1998).

No differences in D_γ or loss of CV binding activity were observed between the two YEP⁺ serotypes regardless of the suspending medium. Statistically significant increases in the rate of CV binding loss were observed starting at the 0.4 kGy and 0.6 kGy doses in strains GERP⁺ and ATCC 51871, respectively. This is in contrast to results of Shenoy et al. (1998) that indicated no decrease of virulence of plasmid-bearing *Y. enterocolitica* following gamma irradiation. In that work six *Y. enterocolitica* colonies were tested for the presence of chromosome and plasmid encoded virulence factors

at each radiation dose. In this study approximately 25,000 *Y. enterocolitica* colonies were screened for the CV binding phenotype. The differences in results obtained between the studies are due to the number of surviving colonies screened and the endpoints tested.

Gamma radiation disrupts cell membrane associated DNA complexes required for plasmid partitioning and active sites for the DNA repair process (Watkins 1980, Khare et al. 1982). Gamma radiation induces DNA strand-breaks, transition mutations, transversion mutations, frameshift mutations, and deletions (Glickman et al. 1980, Raha and Hutchison 1991, Sargentini and Smith 1994, Wijker et al. 1996). Mudgett et al. (1990) observed that gamma radiation induced mutagenesis in *E. coli* started when post-irradiation survival reached 1.5%, or a dose of 0.6 kGy. Wijker et al. (1996) recommended a gamma radiation dose of 0.25 kGy, which decreased survival to 2%, for proper selection and characterization of mutants in *E. coli* strain EC919. CV loss occurred at a radiation dose which produced a two log(10) reduction in viable organism, consistent with radiation doses required for induction of mutation and membrane damage in other studies.

Previous molecular and phenotypic characterization of *Y. enterocolitica* which carry the virulence plasmid, in addition to characterization of *Y. enterocolitica's* radiation sensitivity ($D_\gamma \approx 0.18$ kGy), make it an ideal model system for study of ionizing radiation-induced attenuation of foodborne pathogens. Raw pork can be irradiated to a dose of 1.75 kGy without loss of sensory attributes (Grant and Patterson 1991). Yet *Salmonella* spp., many of which carry virulence plasmids, have D_γ up to 0.51 kGy when suspended in pork (Thayer et al. 1995). Therefore, the 2.55 kGy radiation dose required to produce a five log(10) reduction of viable *Salmonella* in pork, would be detrimental to pork's sensorial qualities. In products such as pork, for which lower radiation doses are appropriate, attenuation, as opposed to elimination of higher D_γ foodborne pathogens, might play a greater role in consumer health protection. Unfortunately, at present, no easy-to-use functional screening assay exists for quantitation of virulence plasmid loss in *Salmonella* spp.

At least 29 plasmid-encoded genes required for assembly of the *Y. enterocolitica* type III secretion channel have been identified and characterized at the molecular level (Cornelis et al. 1998). At least 12 other genes required for Yop's protein translocation and host immune suppression have been mapped to the large virulence plasmid (Cornelis et al. 1998). Future work will include DNA sequence analysis of plasmid encoded genes required for virulence in *Y. enterocolitica* that survive a radiation dose sufficient to reduce the viable population by five log (10). Mutational analysis, combined with virulence studies using *Y. enterocolitica* that survived the irradiation process, will help elucidate the mechanisms by which foodborne pathogens are attenuated following treatment with low-dose ionizing radiation. In addition, CV binding loss rate in actively growing *Y. enterocolitica* will also be determined.

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