Food Research International 45 (2012) 1054–1057

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Growth response of *Salmonella enterica* Typhimurium in co-culture with ruminal bacterium *Streptococcus bovis* is influenced by time of inoculation and carbohydrate substrate

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ARTICLE INFO

Article history:
Received 11 March 2011
Accepted 1 July 2011

Keywords:
Salmonella Typhimurium
Streptococcus bovis
Growth response
Inoculation time
Carbon substrates

ABSTRACT

Streptococcus bovis (*S. bovis*) is a normal inhabitant of the gastrointestinal tract of ruminants. It has a high metabolic rate and short generation time. Under conditions of carbohydrate overload, *S. bovis* can outgrow other ruminal microorganisms and dramatically lower the pH of the rumen. The purpose of this study was to characterize the growth effect on foodborne *Salmonella enterica* Typhimurium (*S.* Typhimurium) in the presence of the ruminal *S. bovis* JB1 isolate under differing incubation conditions. When *S. bovis* and *S.* Typhimurium were inoculated simultaneously and co-cultured together in Luria–Bertani broth, growth inhibition of *S.* Typhimurium was observed. In minimal medium supplemented with different carbon sources, inhibition of growth of *S.* Typhimurium was greatest in glycerol, followed by maltose, trehalose, and glucose. When the inoculation sequence was staggered in the co-culture with an initial inoculation of *S. bovis* followed by a subsequent inoculation with *S.* Typhimurium, growth inhibition of *S.* Typhimurium was greater for all carbon sources. Based on these studies it appears that carbon substrate and time of inoculation influence *S.* Typhimurium growth in the presence of actively growing *S. bovis*.

1. Introduction

Salmonella is a significant hazard to human safety and a difficult challenge for the food industry. Estimated costs associated with *Salmonella* infections are billions of dollars annually in the United States (Voetsch et al., 2004). *Salmonella* is also the foodborne pathogen furthest from its target goal in the Healthy People 2010 Initiative (CDC, 2010). Although *Salmonella* can be acquired from water or pets, up to 95% of salmonellosis cases are associated with foods of animal origin (Mead et al., 1999). The *Salmonella enterica* serovars Enteritidis and Typhimurium are the two most commonly isolated *Salmonella* serovars from cases of human food poisoning in the United States (Schroeder et al., 2005). *Salmonella* illnesses are most commonly associated with the consumption of poultry meat and products, including eggs (Cox, Berrang, & Cason, 2000; Doyle, Kaspar, Archer, & Klos, 2009). *Salmonella* continues to be a problem in part because of the widespread occurrence of the organism in natural reservoirs and intensive husbandry practices (Hardy, 2004).

Streptococcus bovis is a Gram-positive bacterium that is serologically classified as a group D *Streptococcus* (Schlegal, Grimont, Ageron, Grimont, & Bouvet, 2003). *S. bovis* can be present in the rumen of cattle, and may outgrow other rumen organisms if the diet has an abundance of soluble carbohydrates (Hungate, Dougherty, Bryant, & Cello, 1952). This organism has some features that make it suitable as a probiotic in non-ruminants including being fast growing, acid tolerant and it has the ability to break down starch rapidly (Stewart, Flint, & Bryant, 1997). Some strains of *S. bovis* produce bacteriocins (Jarvis, 1967), but *S. bovis* JB1 is a bacteriocin-negative strain. Although *S. bovis* JB1 does not produce bacteriocins, it grows more rapidly than does the bacteriocin producing strain HC5 (Contreras-Govea, Muck, & Russell, 2008).

The ceca of birds contains an ecosystem of organisms that provides a natural means of resisting colonization by enteric pathogens such as *Salmonella* (Ricke, Woodward, Kwon, Kubena, & Nisbet, 2004). The time to establish the microflora of the ceca in chicks can take up to 4 weeks (Mead & Adams, 1975), during which the birds are susceptible to colonization. Probiotics are viable organisms that can be fed to animals that may establish in the gastrointestinal tract and provide some benefit to the animal (Patterson & Burkholder, 2003). Probiotics have been shown to be successful in minimizing the colonization of chicks by *Salmonella* (Stavric, 1992). *S. bovis* has also been previously examined as a potential silage inoculant (Contreras-Govea et al., 2008; Jones, Muck, & Ricke, 1991). Our objective in this study was to evaluate the suitability of *S. bovis*

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doi:10.1016/j.foodres.2011.07.001

strain JB1 as a potential probiotic by determining whether *S. bovis* would limit the growth of *S.* Typhimurium *in vitro*. We also evaluated the influence of carbon source and time of inoculation on inhibition of growth of *S. bovis* and the ability of *S. bovis* to inhibit the growth of *S.* Typhimurium.

2. Materials and methods

2.1. Bacterial strains and culturing

S. bovis strain JB1 was provided by Dr. Michael Cotta (National Center for Agricultural Utilization Research, USDA Agricultural Research Service, Peoria, IL) and *S.* Typhimurium ATCC 14028 was obtained commercially. Cultures were grown in sterile Luria–Bertani Broth (LB; BD Diagnostics, Franklin Lakes, NJ) which was incubated at 37 °C overnight or for 24 h. Solid medium used was Luria–Bertani agar (LBA; BD Diagnostics, Franklin Lakes, NJ).

2.2. Inhibition of S. Typhimurium by S. bovis

Overnight cultures of *S. bovis* and *S.* Typhimurium were diluted with sterile PBS to a concentration of 10^8 CFU/ml. Sterile LB was dispensed into a 96 well microtiter plate, 180 µl per well. Wells were inoculated with a 10 µl aliquot (containing 10^6 organisms) of either *S. bovis* or *S.* Typhimurium alone plus 10 µl of sterile PBS, or with a co-culture containing 10 µl of each bacterium. Plates were incubated at 37 °C for 12 h, with growth measured by culture turbidity at 600 nm every 30 min on a Tecan Infinite M200 microtiter plate reader (Tecan Systems, Inc., San Jose, CA). The studies were repeated to introduce a 6 and 12 h time period of initial *S. bovis* growth before the inoculation of *S.* Typhimurium into the co-cultures.

2.3. Growth rate and lag time

Sets of 3 culture tubes were filled with 9 ml of M9 minimal medium (Davis, Botstein, & Roth, 1980) supplemented with different carbon sources. Each carbon source was added to produce a final concentration of 1.8%. The tubes were inoculated with overnight cultures of *S. bovis* or *S.* Typhimurium diluted with PBS to a concentration of 10^8 CFU/ml. The tubes were incubated at 37 °C for 12 h. Growth was measured by culture turbidity ($\lambda = 600$ nm) hourly. The assay was performed 3 times and the absorbance values were transformed to the natural logarithm and averaged. The growth rate μ (h^{-1}) was obtained by plotting the natural log versus time (Ricke & Schaefer, 1991). The lag phase L (h) was calculated using the formula: $\text{Ln Ab}_t = (t - L)\mu + \text{Ln Ab}_0$.

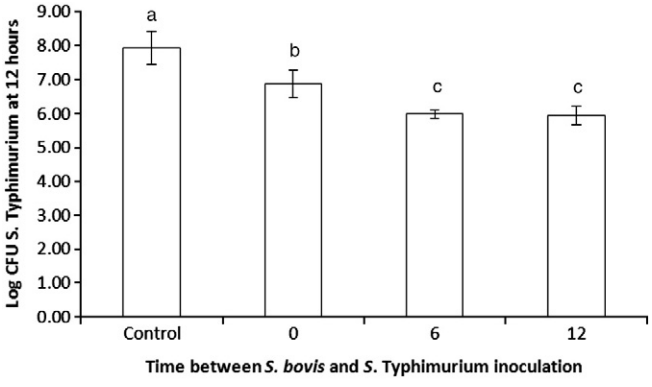

Fig. 1. Effect of time of inoculation of *S.* Typhimurium into *S. bovis* culture in Luria–Bertani broth. Control is *S.* Typhimurium alone. [a–c]Different letters denote significant difference ($p<0.05$) by Student's *t* test.

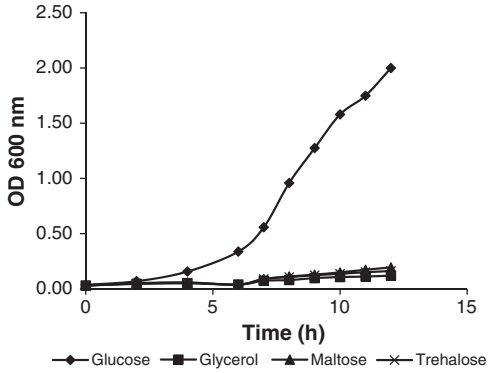

Fig. 2. Growth of *S.* Typhimurium in M9 medium supplemented with 1.8% glucose, glycerol, maltose or trehalose.

2.4. Influence of sugar or time of inoculation on growth inhibition

Overnight cultures of *S. bovis* and *S.* Typhimurium (resistant to both nalidixic acid and novobiocin) were diluted with PBS to a concentration of 10^8 CFU/ml. A minimal medium, M9, was prepared containing various sugars and 9 ml aliquots dispensed into culture tubes (5 tubes/experimental group). Tubes were inoculated with 0.5 ml of the *S. bovis* culture and incubated at 37 °C. A 0.5 ml aliquot of the *S.* Typhimurium suspension was added at 0, 6 and 12 h after the initial *S. bovis* inoculation. After inoculation with *S.* Typhimurium, the tubes were further incubated at 37 °C for 12 h. Samples from the tubes were serially diluted in PBS and plated on both LB agar and Brilliant Green Agar (BG; BD Diagnostics, Franklin Lakes, NJ) supplemented with both nalidixic acid (20 µg/ml) and novobiocin (25 µg/ml). Plates were incubated at 37 °C for 24 h, and differential growth of the two bacteria was determined by comparing the number of colonies on the LB plates (*S. bovis* and *S.* Typhimurium) to the BG with novobiocin and nalidixic acid plates (*S.* Typhimurium only).

2.5. Statistical analysis

All experiments were performed three separate times, with three replicates per experiment. Results were entered into an Excel spreadsheet and averages and standard deviations from the mean were determined using the Excel functions for each treatment. Means were compared using the Student's *t* test with significance assigned at $p<0.05$.

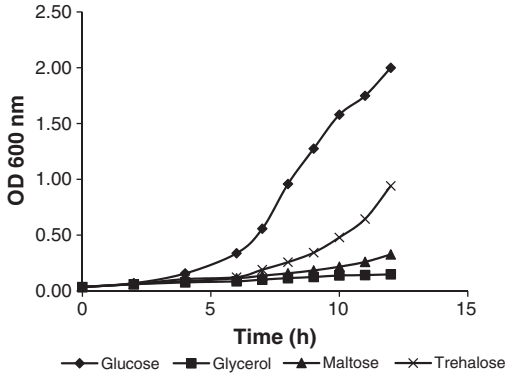

Fig. 3. Growth of *S. bovis* in M9 medium supplemented with 1.8% glucose, glycerol, maltose or trehalose. [a–b]Lag times for glucose and trehalose are significantly different ($p<0.05$) by Student's *t* test.