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Comparison of Growth of Campylobacteriaceae on Media Supplemented with Organic Acids and on Commercially Available Media

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Abstract: Experiments were conducted to compare the ability of a medium supplemented with organic acids to the ability of commercially available, non-selective media to support growth of *Campylobacter* spp. and *Arcobacter butzleri*. Liquid medium was composed of yeast extract-peptone basal broth (BB) that could be supplemented with an organic acid (OA) mixture of fumaric, lactic, malic, and succinic acids (BB + OA). Growth of cultures in BB and in BB + OA was compared to growth in Brucella broth, Mueller-Hinton broth, and Fluid Thioglycollate medium. Growth of *A. butzleri* and 10 of 15 *Campylobacter* isolates was significantly ($P \leq 0.05$) greater in BB + OA than in other broth media, while there was no significant difference in growth of the other 5 *Campylobacter* isolates in BB, BB + OA, Brucella broth, Mueller-Hinton broth, or Fluid Thioglycollate medium. Growth was also compared on agar media composed of BB + OA with Bacto agar and hemin (BA-hemin + OA) and on Remel® Blood Agar. Results indicated that there was no significant difference in the number of cfu's recovered on BA-hemin + OA and on blood agar. Findings indicate that media supplemented with organic acids may be used as alternatives to some currently available commercial media for growing and maintaining Campylobacteriaceae.

Key words: *Campylobacter*, *Arcobacter*, media, organic acids

Introduction

Campylobacter (Frost, 2001) and *Arcobacter* (Houf *et al.*, 2001; Houf *et al.*, 2002) are recognized as major human, foodborne pathogens. The genus *Campylobacter* is composed of strictly microaerophilic bacteria, while the genus *Arcobacter* is composed of bacteria that were previously defined as aerotolerant campylobacters (Zimbro and Power, 2003). Currently, the genera *Campylobacter* and *Arcobacter* are the only members of the family Campylobacteriaceae (Vandamme and De Ley, 1991; Vandamme *et al.*, 1991). Both bacteria are frequently found in the intestinal flora of animals, especially birds. Improperly prepared poultry products are a major source of foodborne infections caused by these bacteria (Houf *et al.*, 2001; Lucia *et al.*, 2004). *Campylobacter jejuni* and *Campylobacter coli* are responsible for 95% of human cases of campylobacteriosis, and *C. jejuni* is currently identified as the major cause of human foodborne bacterial illnesses in developed and developing countries (Friedman *et al.*, 2000). Bacteria in the genus *Arcobacter* are now considered to be emerging foodborne pathogens (Rivas *et al.*, 2004).

Campylobacter and *Arcobacter* exhibit similar phenotypic characteristics, and both bacteria are fastidious with exacting nutritional requirements (Park, 2002). The bacteria do not metabolize carbohydrates, but they may obtain energy by oxidizing amino acids and intermediates of the tricarboxylic acid (TCA) cycle (Stern and Line, 2000). Although Campylobacteriaceae can grow on basal media, their growth is enhanced by the addition of supplements to the media. Defibrinated or lysed blood is a common supplement used in campylobacter media; however, blood-free media are desirable because of the cost and variability in quality of animal blood used as media supplements (Bolton *et al.*, 1984). Many of the media used to culture Campylobacteriaceae also contain oxygen scavengers such as charcoal, ferrous sulphate, sodium metabisulphite, sodium pyruvate, or hemin to protect the bacteria from the toxic effects of metabolic oxygen derivatives (Corry *et al.*, 1995; George *et al.*, 1978; Line, 2001). These oxygen scavengers may be used in various combinations with or without blood.

Recent research has indicated that supplementing a yeast extract-peptone basal medium with the optimum concentration of selected TCA cycle intermediates and/or lactic acid significantly increases the growth of *Campylobacter* spp. *in vitro* (Hinton, 2005). These organic acids are also metabolic by-products of several bacteria found in the native bacterial flora of the intestinal tract of poultry and other animals that serve as reservoirs of Campylobacteriaceae (Schneitz *et al.*, 1981; Szylit *et al.*, 1988). The objective of the present study was to compare the growth of *Campylobacter* spp. and *A. butzleri* in a blood free media supplemented with organic acids and in commercially available media.

Materials and Methods

Bacterial cultures: *Arcobacter* (formerly *Campylobacter*)

butzleri ATCC 49615, *Campylobacter fetus* subsp. *fetus* ATCC 27374, *Campylobacter coli* ATCC 33559, *Campylobacter jejuni* subsp. *jejuni* ATCC 33560, and *Campylobacter jejuni* subsp. *doylei* ATCC 49349 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). (*A. butzleri* ATCC 49615 was identified as *Campylobacter butzleri* ATCC 49615 in the 17th edition of the ATCC catalog that was used as a reference when ordering the culture; however, when the culture arrived it was labeled as *Arcobacter butzleri* ATCC 49615). *C. jejuni* PPMQ2b and *C. jejuni* PPMQ3b were isolated from processed broiler carcasses (Hinton *et al.*, 2004). All other isolates were provided by Dr. Richard Meinersmann of the Agricultural Research Service, Russell Research Center, Athens, GA. Stock cultures of the isolates were maintained by transferring the bacteria, at weekly intervals, to fresh Remel® Blood Agar (Remel, Lenexa, KS) and incubating at 37°C in a BBL GasPak with a CampyPak Plus Hydrogen + CO_2 with Integral Palladium Catalyst for 48 h. After incubation, the plates were stored at 4°C.

Media preparation: Basal broth (BB) media and a mixture of organic acids were prepared separately. A yeast extract-peptone broth was made by dissolving 20 g of yeast extract (Difco Laboratories, Detroit, MI) and 5 g of proteose peptone #3 (Difco) in 900 ml of distilled water. The pH of the solution was adjusted to 7.0 using dilute solutions of NaOH (Spectrum Quality Products, Inc., New Brunswick, NJ) and HCl (Spectrum Quality Products). Nine ml aliquots of the media were dispensed in screw-capped tubes. An organic acid solution (OA) containing 200 mM each of fumaric (Sigma Chemical Co., St. Louis, MO), DL-lactic (Sigma), DL-malic (Sigma), and succinic (Sigma) acids was prepared by mixing the acids in distilled water that was alkalinated with NaOH pellets to maintain a pH of 11-12 until all acids were dissolved. The final pH of the OA solution was then adjusted to 7.0 with dilute solutions of HCl. The yeast extract-peptone broth and the OA mixture were sterilized separately by autoclaving at 121°C for 15 min. Non-supplemented BB was prepared by adding 1 ml of sterile, distilled water to 9 ml of yeast extract-peptone, and BB supplemented with the OA solution (BB + OA) was prepared by adding 1 ml of the OA solution to 9 ml of yeast extract-peptone. Brucella broth, Mueller-Hinton broth, and Fluid Thioglycollate medium, were prepared according to the manufacturers' instructions, dispensed in into test tubes in 10 ml aliquots, and sterilized by autoclaving at 121°C for 15 min. These commercially available broth media were selected because they are recommended by the ATCC for culturing *Campylobacter* and *Arcobacter* isolates ordered from the ATCC culture collection (Gherna and Pienta, 1992).

Basal agar (BA) media was prepared by first, dissolving 12 g of Bacto agar (Difco) in 900 ml of BB broth described above. A hemin solution was made by mixing 0.1 g of hemin chloride, bovine (Sigma) in 2 ml of 1 M NaOH then increasing the total volume of the solution to 10 ml with distilled water. Twenty five mg of hemin (in solution) was added to the agar medium, and the final pH of the medium was adjusted to pH 7.5. BA with hemin (BA-hemin) was autoclaved at 121°C for 15 min, and then cooled to 50°C. BA-hemin with organic acids (BA-hemin + OA) was prepared by adding 100 ml of a sterile 200 mM OA mixture to 900 ml of tempered BA-hemin. The cooled media was dispensed into sterile Petri dishes and allowed to solidify.

Growth of cultures: Stock cultures of *Campylobacter* spp. and *A. butzleri* were streaked onto Remel® Blood Agar plates and incubated in a BBL GasPak Jar with a CampyPak Plus Hydrogen + CO_2 with Integral Palladium Catalyst for 48 h at 37°C. After incubation, cultures were harvested by adding 9 ml of 0.1% Difco Bacto Peptone solution to the surface of the plates and using sterile bacterial cell spreaders to scrape cell growth from the plates. Harvested bacterial suspensions for each of the isolates contained approximately 10^9 cfu/ml. Growth of the bacteria in broth media was examined by adding 0.1 ml of the bacterial suspension to 10 ml of BB, BB + OA, Brucella broth, Mueller-Hinton broth, and Fluid Thioglycollate medium. Test tubes containing the inoculated media were transferred to a MACS VA500 Microaerophilic Workstation (Don Whitley Scientific Limited, West Yorkshire, England) filled with an atmosphere of 82% nitrogen, 10% oxygen, and 8% carbon dioxide. Honeycomb 2 cuvette plates (Labsystems, Inc, Franklin, MA) were placed in the chamber, and 0.1 ml of each inoculated medium was transferred to the wells of the cuvette plates. The cell suspensions were overlaid with 0.1 ml of sterilized, heavy, white mineral oil (Sigma), and the filled Honeycomb 2 plates were removed from the microaerophilic chamber and placed in the incubator tray of a Bioscreen C Microbiology Reader (Thermo Electron Corp., West Palm Beach, FL) that was operated by a computer with Growth Curves Software, v. 2.28 (Transgalactic Ltd., Helsinki, Finland). Cultures were incubated in the Microbiology Reader at 37°C for 48 h, and absorbance of the cultures was measured with the wide band (420-580 nm) filter. Each experiment was replicated 5 times.

To compare the recovery of the isolates on agar media, 48 h cultures were harvested from Remel® Blood Agar plates as described above, and serial dilutions of the cultures were spread plated on BA-hemin + OA and on Remel® Blood Agar plates. Plates were transferred into the MACS VA500 Microaerophilic Workstation and incubated at 37°C for 48h, and cfu were counted after incubation. Each experiment was replicated 3 times.

Table 1: Absorbance[1] of *Arcobacter butzleri* and *Campylobacter* spp. after incubation in broth media at 37°C for 48 h

Isolate Name	Basal Broth (BB)	Basal Broth (BB) + OA[2]	Brucella Broth	Mueller-Hinton Broth	Fluid Thio-glycollate
A. butzleri ATCC 49616	0.44^{c}±0.02	0.49^{d}±0.03	0.20^{b}±0.01	0.12^{a}±0.02	0.17^{b}±0.01
C. jejuni PPMQ2B	0.38^{c}±0.02	0.47^{d}±0.02	0.33^{c}±0.06	0.27^{b}±0.03	0.16^{a}±0.01
C. jejuni PPMQ3B	0.33^{c}±0.04	0.49^{d}±0.04	0.44^{d}±0.02	0.26^{b}±0.03	0.17^{a}±0.01
C. jejuni AMRU 67-8	0.41^{b}±0.04	0.52^{b}±0.02	0.46^{b}± 0.10	0.48^{b}±0.11	0.24^{a}±0.02
C. jejuni AMRU 111-3	0.34^{b}±0.12	0.61^{c}±0.09	0.36^{b}± 0.06	0.40^{b}±0.05	0.17^{a}±0.02
C. jejuni AMRU 127-2	0.12^{a}±0.01	0.66^{c}±0.18	0.38^{b}± 0.13	0.37^{b}±0.05	0.27^{ab}±0.03
C. jejuni AMRU 129-25	0.37^{a}±0.02	0.68^{b}±0.12	0.48^{a}±0.06	0.45^{a}±0.09	0.36^{a}±0.05
C. jejuni AMRU 144-3	0.48^{b}±0.01	0.66^{c}±0.05	0.50^{b}±0.02	0.49^{b}±0.07	0.29^{a}±0.03
C. fetus subsp. *fetus* ATCC 27349	0.29^{b}±0.01	0.73^{a}±0.01	0.38^{c}±0.01	0.42^{d}±0.02	0.19^{a}±0.01
C. jejuni AMRU 1997-8	0.56^{c}±0.05	0.56^{c}±0.02	0.45^{b}±0.03	0.60^{c}±0.01	0.17^{a}±0.01
C. coli ATCC 33559	0.26^{ab}0.02	0.53^{d}±0.06	0.31^{b}± 0.02	0.44^{c}±0.06	0.23^{a}±0.02
C. jejuni ATCC 33560	0.10^{a}±0.02	0.50^{d}±0.03	0.15^{c}±0.02	$0.13^{b,c}$±0.02	0.08^{a}±0.03
C. jejuni AMRU 48100	0.42^{c}±0.02	0.48^{c}±0.02	0.41^{c}±0.08	0.31^{b}±0.06	0.17^{a}±0.02
C. jejuni AMRU 63915	0.30^{a}±0.09	0.54^{b}±0.07	0.36^{b}±0.06	0.41^{a}±0.08	0.09^{a}±0.01
C. jejuni AMRU 86005	0.21^{ab}+ 0.05	0.55^{c}±0.13	0.24^{b}±0.05	0.09^{a}±0.05	0.11^{ab}±0.04

[1]Values are averages ± standard deviation. n = 5.

[2]Media supplemented with organic acid mixture of 20 mM fumaric, lactic, malic, and succinic acids.

[a-e]Within rows, different superscripts indicate significant differences in the absorbance of cultures grown in different media.

Statistical analysis: Data were analyzed using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA). One-Way Analysis of Variance (ANOVA) was used to determine differences in group means of absorbance of bacterial cultures grown in different broth media. When ANOVA detected significant differences in group means, the Tukey-Kramer test was used to determine significant differences in group means. The paired t-test was used to determine significant differences in the number of cfu/ml recovered on BA-hemin + OA and on Remel® Blood Agar plates. All significant differences were determined at $P \leq 0.05$.

Results

Growth of cultures in broth media: In broth media, growth of most of the Campylobacteriaceae cultures was significantly greater in BB + OA than in BB, Brucella broth, Mueller-Hinton broth, or Fluid Thioglycollate medium (Table 1). After 48 h, the absorbance of cultures of *A. butzleri* and cultures of 10 of 15 of the *Campylobacter* isolates grown in BB+OA was significantly higher than the absorption of cultures grown in BB, Brucella broth, Mueller-Hinton broth, or Fluid Thioglycollate medium. Although there was no significant difference in the absorbance of cultures of *C. jejuni* PPMQ3 or *C. jejuni* AMRU 63915 grown in BB + OA or Brucella broth, the absorbance of cultures grown in these two media were significantly higher than the absorbance of cultures grown in the other 3 media. Likewise, there was no significant difference in the absorbance of cultures of *C. jejuni* AMRU 86119 grown in BB or BB + OA, but the absorbance of cultures grown in these media was significantly higher than the absorbance of isolates grown in commercially available media. Furthermore, although there was no difference in the absorbance of cultures of *C. jejuni* AMRU 1997-8 grown in BB, BB+OA, or Mueller-Hinton broth, the absorbance of cultures grown in these media were significantly higher than the absorbance of cultures grown in Brucella broth or Fluid Thioglycollate medium. Similarly, the absorbance of cultures of *C. jejuni* AMRU 48100 cultures grown in BB, BB + OA, or Brucella broth was significantly higher than the absorbance of cultures grown in Mueller-Hinton broth or Fluid Thioglycollate medium. Finally, the absorbance of *C. jejuni* AMRU 67-8 cultures grown in Fluid Thioglycollate medium was significantly lower than the absorbance of these cultures grown in either of the other media.

Recovery of cultures on agar media: On agar media, results indicated that there was no significant difference in the number of cfu/ml recovered from any of the Campylobacteriaceae cultures plated on BA-hemin + OA or on Remel® Blood Agar (Table 2). (BA + hemin agar medium was not included in the study because preliminary experiments indicated that growth of Campylobacteriaceae in BB+hemin was generally significantly less than other media used in the study). Approximately 10^9 cfu/ml of each isolate was recovered on both agar media.

Discussion

Findings of studies conducted with broth media indicate that growth of most isolates of *A. butzleri*, *C. jejuni, and C. coli* is significantly greater in BB+OA than in the commercially available broth media used in the present study. Additionally, the level of recovery of Campylobacteriaceae on agar media supplemented with organic acids was no different from the level of recovery on media supplemented with blood. Yeast extract and peptone in the basal medium served as a

Table 2: Log colony-forming-units/ml *Arcobacter butzleri* and *Campylobacter* spp. recovered on Remel® Blood Agar and Basal Agar-Hemin+OA incubated at 37°C for 48 h[1,2]

Isolate	Remel® Blood Agar	Basal Agar-Hemin+OA[3]
A. butzleri ATCC 49616	9.03±0.23	9.04±0.04
C. jejuni PPMQ2B	9.61±0.13	9.62±0.01
C. jejuni PPMQ3B	9.71±0.18	9.79±0.05
C. jejuni AMRU 67-8	9.94±0.05	9.55±0.52
C. jejuni AMRU 111-3	9.64±0.11	9.45±0.11
C. jejuni AMRU 127-2	9.83±0.09	9.81±0.10
C. jejuni AMRU 129-25	9.71±0.20	9.62±0.08
C. jejuni AMRU 144-3	9.99±0.06	9.73±0.17
C. fetus subsp. *fetus* ATCC 27349	9.40±0.25	9.59±0.07
C. jejuni AMRU 1997-8	9.69±0.10	9.60±0.08
C. coli ATCC 33559	9.47±0.07	9.46±0.06
C. jejuni ATCC 33560	8.97±0.11	8.84±0.06
C. jejuni AMRU 48100	9.64±0.10	9.54±0.11
C. jejuni subsp. *doylei* ATCC 49349	9.45±0.08	9.22±0.10
C. jejuni AMRU 86005	9.39±0.19	9.30±0.13

[1]Values are averages ± standard deviation. n = 3.
[2]There was no significant difference ($P < 0.05$) in the number of cfu/ml recovered on blood agar and
[3]Media supplemented with 25 mg/l of hemin and organic acid mixture (20 mM fumaric, lactic, malic, and succinic acids).

source of amino acids, vitamins, and other growth factors (Vandamme and De Ley, 1991) that support growth of Campylobacteriaceae. Since Campylobacteriaceae can produce energy by oxidizing some intermediates of the TCA cycle (Stern and Line, 2000), the mixture of organic acids added to the media probably provided an additional energy source that stimulated the growth of the bacteria. Earlier research has indicated that supplementing media with the optimum concentration of either one of these TCA intermediates or lactic acid can significantly increase the growth of *Campylobacter* spp. (Hinton, 2005). Other research has also shown that the metabolic activity of the intestinal anaerobe, *Veillonella*, is greater when the bacterium is cultured in a media supplemented with succinic and lactic acid than in media that is not supplemented with these organic acids (Hinton and Hume, 1995). The metabolism of Campylobacteriaceae may be similar to the metabolism of *Veillonella* and other intestinal, non-carbohydrate utilizing bacteria that are able to grow by metabolizing TCA intermediates and lactic acid. Some TCA intermediates and lactic acid are produced in the intestinal tract of mature animals by the native microbial flora of the animals (Schneitz *et al.*, 1981) and are utilized by other bacteria in the native microflora (Hinton *et al.*, 1993). Since campylobacter colonization occurs more readily in poultry with a mature intestinal microflora than in young chicks (Lindblom *et al.*, 1986), the presence requisite concentrations of organic acids produced by a mature microbial flora may be one of the factors that increase the susceptibility of mature poultry to colonization by Campylobacteriaceae. Because of some of the disadvantages of using blood as a media supplement, a blood-free medium that supports the growth of these important foodborne pathogens would be a useful tool for scientists studying these bacteria. Agar and broth media supplemented with organic acids may serve as practical alternatives to some commercially available media currently used for the growth and maintenance of cultures of these important foodborne pathogens.

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