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Survival *Yersinia enterocolitica* in Ground Pork Meat in Different Packages

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In this study the influence of gradual increase of carbon dioxide concentration (percentage by volume in air) on *Yersinia enterocolitica* counts was examined the during packaging and storage of ground pork meat artificially contaminated with these bacteria was evaluated. Ground meat was packaged under customary conditions using vacuum and modified atmospheres with various carbon dioxide percentages (MAP 1 consist of was 20% O_2, 50% CO_2 and 30% N_2, MAP 2 was 20% O_2, 30% CO_2 and 50% N_2). The packs were storage at 4±1 ºC for 12 days. During the entire storage time, counts of *Y. enterocolitica* were determined by plate method for direct plate counts. Also, microbiological shelf life of the stored pork meat was assessed by *Enterobacteriaceae* and total aerobic bacteria plate counts. *Y. enterocolitica* counts were not significantly different ($p>0.01$; $p>0.05$) in the pork under various packaging, but it was significantly different ($p>0.01$; $p>0.05$) between days of comparing in the same packs. Packaging with high CO_2 concentration had inhibitory effect on the grown of *Y. enterocolitica* and *Enterobacteriaceae*.

Key words: *Yersinia enterocolitica*, pork meat, modified atmosphere, vacuum-packed

Y. enterocolitica is psychrotrophic, gram negative, facultative anaerobic zoonotic bacterium belonging to the family *Enterobacteriaceae*[1]. In 2007, 8.792 cases of yersiniosis were reported in humans in the European Union, making the zoonotic agent *Yersinia* the third most important cause of enteritis in humans after *Campylobacter* and *Salmonella*[2]. *Y. enterocolitica* is the most common species reported in human cases, being isolated from 93.8% of all confirmed cases[3].

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Y. enterocolitica is thought to be a significant food-borne pathogen, even though pathogenic strains have seldom been isolated from foods. Pigs are assumed to be the main reservoir of pathogenic *Y. enterocolitica* because pathogenic strains of this microorganism are frequently isolated only from this animal species so far[4]. Several domestic animals like dogs, cats, cows, sheep, and horses and several wild[5] animals like rodents (mainly mice), monkeys, deer, and foxes have also been incriminated as potential reservoirs[6]. Further evidence of the link between pigs, pork carcasses and products is presented in the „Scientific Opinion on the public health hazards to be covered by inspection of meat" (EFSA Panels on Biological Hazards (BIOHAZ)), on Contaminants in the Food Chain (CONTAM), and

on Animal Health and Welfare (AHAW)[3]. *Y. enterocolitica* survives well on chilled carcasses including those subject to blast chilling[7]. Optimum growth temperature for these bacteria is 28-29 °C. They are also capable to growth at -2 ºC[8] and their growth on meat under chilled conditions has been reported[9]. However, the literature is contradictory regarding the multiplication of human pathogenic *Y. enterocolitica* in meat during conventional cold storage.

In recent years, *Y. enterocolitica* has been the third most common cause of bacterial food borne disease in many European countries, with 7.017 confirmed cases in the EU in 2011[10]. This pathogen is readily isolated from tonsil of pigs at slaughter[11]. Contamination of meat with *Y. enterocolitica* may take place during slaughtering and processing of carcasses[12]. A particular public health concern is the ability of *Y. enterocolitica* to refrigeration temperatures[11].

The geographical distribution of *Y. enterocolitica* is diverse. *Y. enterocolitica* has more than 50 distinct serotypes (on the basis of antigenic variations in cell wall lipopolysaccharide), and few of them are pathogenic. The primary infectious serotype in the USA is O:8 followed by O:3, O:5,27, O:13a, 13b, O:20, O:9, and so forth[13]. Serotype O:3 is the most frequently isolated type in humans in Europe[14]. In China, serotype O:3 is primarily found in infections followed by O:9, and O:8[15]. Furthermore, various serotypes demonstrate geographical specificity; for example, the predominant serotype in Australia, Europe, and Canada is O:3[16], O:8 in Japan[17] and O:9 in Scandinavia, The Netherlands[18].

The most common manifestation of *Y. enterocolitica* infection is gastroenteritis, which is usually self-limiting, resulting in diarrhea, mild fever and abdominal pain and sometimes also, reactive arthritis. Numbers of studies have shown that human pathogenic *Y. enterocolitica* is able to multiply in foods kept chilled under storage, and might, even compete successfully with the micro-organisms usually found in food[19].

Modified atmosphere packaging (MAP) of raw meat is very common and widely used by meat industry because it increases the shelf life of raw meat product when coupled with refrigerated storage[20]. Oxygen and carbon dioxide is common mix used for packaging of raw meat. The oxygen allows for formation of oxymyoglobin creating a more desirable pink or red colour of raw pork, while CO_2 suppresses the bacterial growth. Increased levels of CO_2 (20%-40%) in refrigerated storage have shown to inhibit microbial population especially the growth of gram-negative bacteria by increasing their lag phase[20].

The aim of this study was to examine the effect of modified atmospheres with different concentrations of carbon dioxide and vacuum packaging on survival *Y. enterocolitica*.

MATERIALS AND METHODS

Bacterial strain and preparation of inoculim

Y. enterocolitica subsp.*enterocolitica* ATCC® 9610™ (www.atcc.org) was used for the inoculation studies. The bioserotype of *Y. enterocolitica* strain was biotype 1 serotype 0:8. A pure culture of *Y. enterocolitica* strain was grown in brain heart infusion broth (Merck, Germany) at 30 ºC for 24h to stationary phase. At stationary phase, *Y. enterocolitica* reached a density of approximately 10^8 CFU ml^{-1} in the broth. The incubated broth was diluted with physiological NaCl-peptone water (0.75%), and the dilution was used immediately for the inoculation of the ground pork meat samples with target inoculums of 10^4 CFU g^{-1}.

Preparation of meat, meat packaging and storage

For this study, we used about 4 kg raw pork meat from slaughterhouse and placed in sterile plastic bags and minced. The ground pork meat was collected in sterile plastic bags, weighing 100±5 g. The surface of each sample was inoculated with 1 ml of the inoculums applied by drops, and the inoculums was mixed into the meat a sterile spatula. A packing machine "Variovac" (Variovac Primus, Zarrentin, Germany) was used for packaging of samples. Samples were packed in a foil OPA / EVOH / PE foil (oriented polyamide / ethylene vinyl alcohol / polyethylene Dynopack, POLIMOON, Kristiansand, Norway), with low permeability to gas. The degree of permeability to O_2 - 3.2 cm^3/m^2 / day at 23 °C, N_2 - 1 cm^3/m^2 / day at 23 °C, the degree of permeability to CO_2 - 14 cm^3/m^2 / day at 23 °C, and to water vapor 15 g/m^2 / day at 38 °C. The inoculated ground meat was packaged in different modified atmospheres (MAP 1 and MAP 2) and vacuum-packed. MAP 1 was 20% O_2,

50% CO_2 and 30% N_2, MAP 2 was 20% O_2, 30% CO_2 and 50% N_2. The packaged pork meat stored at the 4±1 ºC for 12 days. Six packs per packaging variation were analyzed on storage 0, 3, 6, 9 and 12 day. The pH value was measured with pH Meter "Testo 205" (Testo AG, Lenzkirch, Germany).

Microbiological analysis

From each sample, 10 g was transferred to a stomacher bag (Sampling, stomacher 400 classic bags, Vicor), 90 ml MRD (Maximum Recovery Diluent, Merck, Germany) was added and content was homogenized for 1 min with a stomacher blender (Stomacher 400Circulator, Seward, UK). For enumeration of *Enterobacteriaceae* 1 ml of the appropriate 10-fold serial dilution was inoculated into VRBG agar (Violet red bile glucose, Merck, Germany). The VRBG plates were incubated at 30 ºC for 24h. All purple colonies due to rapid fermentation of glucose surrounded by purple haloes of precipitated bile salts were counted[11]. Total aerobic bacteria counts were determined by pour plate technique[21], in PCA (Plate Count Agar, Merck, Germany), incubated at 35ºC for 48h. For enumeration of *Y. enterocolitica* we used CIN (Yersinia selective agar base CM0653, Oxoid, UK and Yersinia selective supplement, SR0109, Oxoid, UK), incubated 30ºC for 24h. *Enterobacteriaceae*, total aerobic mesophiles and *Y. enterocolitica* counts were determined after 0, 3, 6, 9 and 12 days of storage.

Statistical analyses

Statistical analysis of the results was elaborated using software GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

RESULTS AND DISCUSSION

Initial pH value of the pork was 6.04±0.01 (Table 1). No significant differences occured in pH value between vacuum, MAP 1 and MAP 2 during 0 and 3 day, but it was found significant differences from 6, 9 and 12 day ($p<0.01$; $p<0.05$). During packaging of meat and meat products in a gas mixture comprising carbon dioxide reduction in the pH value, on the one hand as a consequence of the solubility of carbon dioxide in the tissue, where the carbonic acid formed, which consequently leads to lowering of the pH, but also due to the antimicrobial activity of CO_2, which results in the inhibition of growth of microorganisms due to microbial activity which has been a build-up of the base components that would otherwise be originated by microbial degradation[20].

Table 1. pH value during storage time

Group	Day of storage (X ± Sd)				
	0.	3.	6.	9.	12.
Vaccum	6.04±0.01	5.85±0.01	5.81^{AB}±0.01	5.67^{AB}±0.02	5.60^{a}±0.01
MAP 1	6.04±0.01	5.87±0.01	5.71^{A}±0.02	5.97^{AC}±0.01	5.96^{a}±0.07
MAP 2	6.04±0.01	5.86±0.01	5.73^{B}±0.02	5.80^{BC}±0.01	5.89±0.01

Legend- $^{A-C}$ statistical significance of $p\leq0.01$; ^{a} statistical significance of $p \leq0.05$

Enterobacteriaceae

Results of *Enterobacteriaceae* are shown in Table 2. The presence of *Enterobacteriaceae* in meat and meat products are examined in order to assess the general hygienic status of meat. Same authors recommend that the average number of *Enterobacteriaceae* can be used as criteria in assessing the sustainability of meat[8]. Some of the *Enterobacteriaceae* are of interest to public health while others have commercial importance because of its ability to cause malfunction of meat and meat products during storage at refrigeration temperatures. Number of *Enterobacteriaceae* increased from all days and in all packaging variations (vacuum, MAP 1 and MAP 2). There are significant differences ($p<0.01$; 0.05) between storage time (0, 3, 6, 9 and 12 day) in vacuum packaging. Also, there are significant differences ($p<0.01$) in MAP 1 during storage time. Number of *Enterobacteriaceae* in MAP 2 increased from all day of storage and was highest compared to the vacuum and MAP 1.

Table 2. Number of *Enterobacteriaceae* in vacuum, MAP 1 and MAP 2 during storage (log CFU/g)

Storage time (days)	Group		
	Vacuum (X± Sd)	MAP 1	MAP 2
0	7.25^{ABC}±0.14	7.25^{AB}±0.14	7.24^{ABC}±0.15
3	7.23^{DEF}±0.05	7.25^{CD}±0.05	7.14^{DEF}±0.04
6	7.61^{ADa}±0.06	7.34^{E}±0.05	7.61^{AD}±0.06
9	7.54^{BE}±0.05	7.47^{AC}±0.06	7.59^{BE}±0.06
12	7.60^{CFa}±0.06	7.58^{BDE}±0.06	7.73^{CF}±0.06

Legend- $^{A-F}$ statistical significance of p≤0.01; ^{a} statistical significance of p≤0.05

Hudson *et al*.[22] stated that to inhibit the growth of psyhrotrophic bacteria it is necessary an atmosphere with more than 75% CO_2. In our study, number of *Enterobacteriaceae* was the least in MAP 1 (7.58±0.06 log CFU/g), where concentration of CO_2 was 50%. According to this research, it can be concluded that MAP with higher concentrations of CO_2 is the best choice for pork meat, because CO_2 has antibacterial effect.

Total aerobic bacteria

The growth date for total aerobic bacteria in the pork meat under various atmosphere and vacuum are presented in Table 3.

Table 3. Number of total aerobic bacteria in vacuum, MAP 1 and MAP 2 during storage (log CFU/g)

Storage time (days)	Group		
	Vacuum (X± Sd)	MAP 1	MAP 2
0	8.19^{A} ±0.07	8.19^{ABC}±0.08	8.19^{A}±0.07
3	8.11^{BC}±0.09	8.22^{DEF}±0.02	8.18^{B}±0.05
6	8.14^{DE}±0.04	8.34^{ADGa}±0.05	8.25^{C}±0.04
9	8.29^{BD}±0.07	8.50^{BEG}±0.06	8.26^{D}±0.05
12	8.39^{ACE}±0.05	8.45^{CFa}±0.05	8.39^{ABCD}±0.06

Legend- $^{A-F}$ statistical significance of p≤0.01; ^{a} statistical significance of p≤0.05

After 6 days of storage in vacuum the number of total aerobic bacteria increased significantly (p<0.01) by 8.14±0.04 log CFU/g, reaching a maximum of 8.39±0.05 log CFU/g at the and of storage period (day 12). However, Kakouri and Nychas[23], Hudson *et al*.[22] and Bell *et al*.[24] found that aerobic bacteria needed approximately 10 days to achieve these counts in same atmosphere in different products, i.e. red meat and smoked cod. Zeitoun *et al*.[25] detected decreased total aerobic bacteria packaged in MAP with 90/10 CO_2/O_2. According to table 3, total aerobic bacteria grew slower under MAP 2. The different behavior observed can be explained by use of O_2 combined with CO_2. O_2 favors the growth of aerobic bacteria. Moreover, N_2 does not have the same effects[26].

Effect of the different MAPs on growth of *Y. enterocolitica*

The minced pork meat was examined before inoculation and was free of *Y. enterocolitica*. Growth of *Y. enterocolitica* is present as a function of storage time at 4±1 ºC in Table 4.

In fresh pork meat packed under vacuum and MAP number of *Y. enterocolitica* was maintained. In this study it was found that *Y. enterocolitica* grew faster in vacuum packed than in MAP. Bodnaruk and Draughon[27] have reported that *Y. enterocolitica* not grow in pork in vacuum packed during storage for 5 ºC.

Table 4. Number of *Y. enterocolitica* in vacuum, MAP 1 and MAP 2 during storage (log CFU/g)

Storage time (days)	Group		
	Vacuum (X± Sd)	MAP 1	MAP 2
0	6.34^{aABC}±0.21	6.34^{aABC}±0.21	6.34^{ABCD}±0.21
3	6.52^{aDE}±0.05	6.11^{aDEF}±0.05	5.81^{AEFG}±0.04
6	6.67^{AF}±0.04	6.64^{ADG}±0.05	7.10^{BEHI}±0.03
9	6.81^{BDG}±0.04	6.61^{BEH}±0.07	7.67^{CFHa}±0.05
12	7.56^{CEFG}±0.06	7.32^{CFGH}±0.07	7.57^{DGIa}±0.04

Legend- $^{A-F}$ statistical significance of p≤0.01; ^{a} statistical significance of p≤0.05

In pork with pH value below 5.8 that was packaged in 100% CO2 and storage at 4 ºC, the growth of *Y. enterocolitica* was suppressed[28]. However, in pork packaged under the same conditions but with an initial pH value upward of 6.0 growth of *Y. enterocolitica* was not inhibited[27]. Manu- Tawiah *et al*.[29] also observed rapid growth of this pathogen in fresh pork chops (pH 6.0) stored in carbon dioxide-enriched atmospheres of 40% CO_2 , 0% O_2 and 60% N_2.

Packaging the fresh pork meat under high CO_2 atmospheres (MAP 1) resulted reduction in *Y. enterocolitica* by the end of storage time (Figure 1.). Number of *Y. enterocolitica* increase in the vacuum packaging. Highest increase in number of *Y. enterocolitica* was in MAP 2, where was found 7.57±0.04 log CFU/g 12 day of storage.

The effect of high oxygen atmospheres on *Y. enterocolitica* growth has not been described

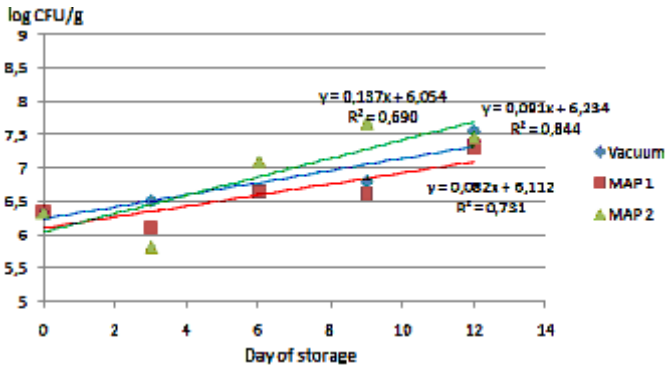

Fig. 1. Effect of different modified atmosphere and vacuum on the survival of *Y. enterocolitica*

conclusively, but there is information indicating that oxygen has an additional inhibitory affect on the growth of *Y. enterocolitica*[30].

Same authors have reported inhibition of *Y. enterocolitica* by the natural microflora in mixed cultures in raw pork[28]. These reports support the hypothesis that the inability of *Y. enterocolitica* to multiply appeared to be associated with the presence of competitive microflora[28]. Inhibition was not the consequence of depletion in essential nutrients or an unfavorable change in pH value. Similar mechanisms can explain the slight decrease of *Y. enterocolitica* in the MAP 1.

The infective dose of *Y. enterocolitica* in food is still unknown[31]. Bhaduri and Turner-Jones[32] stated that the virulent characteristics of this microorganism remain the same, even in anaerobiosis and mixtures of CO_2 and other gases, being able to cause foodborne disease. The study of *Y. enterocolitica* in MAP food becomes significant to public health, provided that this pathogen can be present in food and is able to grow in those conditions, even in low levels[28]. Therefore, a prevention strategy along the food

chain is necessary. According to Fredriksson-Ahomaa *et al*.[33] improvements in the slaughtering process may reduce the *Y. enterocolitica* incidence in pork meat.

CONCLUSIONS

In conclusion, it has been demonstrated that the *Y. enterocolitica* survived in pork stored under vacuum and MAP at a low temperature. Pork microflora had no discernible effect on the survival of *Y. enterocolitica.* Furthermore, the survival of *Y. enterocolitica* for an extended period of time in pork and transmission of the pathogen to other foods may be a critical issue in efforts to insure food safety.

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