JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Carnobacterium Species: Effect of Metabolic Activity and Interaction with *Brochothrix thermosphacta* on Sensory Characteristics of Modified Atmosphere Packed Shrimp

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The importance of carnobacteria as spoilage microorganisms or potential protective cultures in food is not resolved, and little is known about their metabolism during growth in specific products. This study used chromatographic techniques including GC-MS and HPLC to evaluate the spoilage metabolism of *Carnobacterium divergens*, *Carnobacterium maltaromaticum*, and *Carnobacterium mobile*. Metabolic activity was studied in cooked and peeled modified atmosphere packed (MAP) shrimp at 5 °C as carnobacteria has been anticipated to contribute to spoilage of shrimp products. *C. divergens* and *C. maltaromaticum* caused sensory spoilage of shrimps and generated ammonia, tyramine, and various alcohols, aldehydes, and ketones. The effects of *Carnobacterium* species on the growth and metabolism of *Brochothrix thermosphacta* were also evaluated, but metabiosis between the two groups of bacteria was not observed. *C. mobile* and a specific cluster of *C. maltaromaticum* isolates (cluster L) did not cause sensory spoilage of shrimp.

KEYWORDS: ***Carnobacterium*; *Brochothrix thermosphacta*; spoilage metabolites; GC-MS; HPLC**

INTRODUCTION

Carnobacterium species have previously been studied both as protective cultures, to inhibit pathogenic and spoilage microorganisms, and as potential spoilage bacteria in chilled seafood and meat products. In these products, *Carnobacterium divergens* and/or *Carnobacterium maltaromaticum* frequently dominate the natural spoilage microbiota, particularly in frozen and thawed products that are vacuum packed (VP) or modified atmosphere packed (MAP) (*1*). For seafoods, high concentrations of *C. maltaromaticum* and/or *C. divergens* have been anticipated to contribute to the sensory spoilage of cooked and brined MAP shrimp at 5−8 °C, frozen and thawed MAP salmon steaks at 2 °C, and high-pressure-processed VP squid mantle at 4 °C (*2*−*4*). Furthermore, Mejlholm et al. (*5*) recently showed that the sensory spoilage characteristics of chilled cooked and peeled MAP shrimp, that is, wet-dog, chlorine-like, and sour off-flavors, were caused by the combined activity of *C. maltaromaticum* and *Brochothrix thermosphacta*. Clearly the effect of carnobacteria and their metabolites on seafood spoilage must be taken into account when they occur in high concentrations as part of the natural spoilage microbiota or when they are studied as protective cultures to be added to foods.

C. divergens and *C. maltaromaticum* are known to metabolize amino acids and to produce tyramine, ammonia, ornithine, acetate, diacetyl/acetoin, methylbutanal, methylbutanol, methylpropanal, methylpropanol, and pentanedione as recently summarized (*1*, *6*). For cold-smoked salmon the metabolites formed by *C. maltaromaticum* have been determined using GC-MS, but in that product the detected metabolites did not result in sensory spoilage (*6*). However, for other seafoods including shrimp products the metabolic activity and sensory importance of carnobacteria remain little studied.

The objectives of the present study were to evaluate the metabolic activity and spoilage potential of *Carnobacterium* species. This was evaluated using shrimp meat as these bacteria seem to be important in different shrimp products. Cooked and peeled MAP shrimps was studied, and this particular product has been shown previously to spoil due to activity of *C. maltaromaticum* and *B. thermosphacta* (*5*). Spoilage and metabolic activity of *C. divergens*, *C. maltaromaticum*, and *Carnobacterium mobile* were studied for each species alone and in coculture with *B. thermosphacta* in shrimp meat. In addition, a specific cluster of *C. maltaromaticum* (cluster L) was studied as it was found recently to inhibit the growth of both *B. thermosphacta* and *Listeria monocytogenes* (*1*) and, therefore, has potential as a protective culture. Activity of the studied carnobacteria was evaluated using sensory, microbiological, and analytical chemical methods including GC-MS and HPLC analyses.

MATERIALS AND METHODS

Inoculation, Packaging, and Storage of Shrimp. Growth and metabolism of *Carnobacterium* in cooked and peeled shrimp were studied for (i) mixtures of isolates from different *Carnobacterium*

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10.1021/jf053017f CCC: $33.50
Published on Web 04/19/2006

Table 1. Overview of the Nine Studied Sub-batches of Cooked and Peeled MAP Shrimp (Sub-batches Were Inoculated with Different Mixtures of Bacteria to an Initial Concentration of ~10^5 CFU/g)

inoculation	none	*Carnobacterium* spp.				*B. thermosphacta* and			
species		*C. divergens*	*C. maltaromaticum*		*C. mobile*	*B. thermosphacta*	*C. divergens*	*C. maltaromaticum*	*C. mobile*
cluster		G	H	L	S and X		G	H	S and X
isolates		N14	F29-1	F46-1	5kb-5[a]	A1-16[b]	9 isolates from Bt and Cd	10 isolates from Bt and Cm-H	7 isolates from Bt and Cmo
		ML1-94	FM-C4	ML1-97	5kb-6[a]	B1-17[b]			
		3b-5bA	N1		5kb-7[a]	B1-18[b]			
		N20	ML1-95			C1-18[b]			
		Ovb-3[a]	OB6-1b						
			R1.7						
treatment code	control	Cd	Cm-H	Cm-L	Cmo	Bt	Bt+Cd	Bt+Cm-H	Bt+Cmo

[a] Isolate originally obtained from cooked and brined MAP shrimp (*1*, *2*). [b] Isolate originally obtained from cooked and peeled MAP shrimp (*5*).

species, (ii) a mixture of *B. thermosphacta* isolates, and (iii) mixtures of *Carnobacterium* isolates in cocultures with a mixture of isolates of *B. thermosphacta*. Individually quick frozen (IQF) shrimp (*Pandalus borealis*) from the North Atlantic Ocean were used for these experiments. The shrimps were caught, cooked, peeled, frozen, and glazed in Greenland and supplied in the frozen state to the Danish Institute for Fisheries Research (DIFRES), Lyngby, Denmark. At DIFRES the shrimp were kept at −22 °C until the start of the experiments. Shrimp, with an average weight of ~2 g per cooked and peeled shrimp, were randomly divided into nine sub-batches (~3 kg of shrimp per sub-batch) and inoculated with mixtures of *C. divergens*, *C. maltaromaticum*, *C. mobile*, and *B. thermosphacta* according to **Table 1**. All isolates were obtained from seafood as described by Laursen et al. (*1*) for *Carnobacterium* and by Mejlholm et al. (*5*) for *B. thermosphacta*. Shrimp were inoculated with 1% (v/w) of solutions containing mixtures of isolates resulting in an initial concentration of ~10^5 colony-forming units (CFU)/g. Isolates were precultured individually at 20 °C in APT broth (Difco 265510), mixed, washed to remove nutrients from preculture medium, diluted in a 1.6% (v/w) NaCl solution, and added to the shrimp, which were then manually tumbled to ensure an even distribution of bacteria in sub-batches of shrimp. Control samples were inoculated with 1% (v/w) of a 1.6% (v/w) NaCl solution without bacteria. Inoculated shrimp (~115 g portions) were packed with a gas/shrimp ratio of 4:1 as previously described in a modified atmosphere initially containing 50% CO_2, 30% N_2, and 20% O_2 (*5*) and then stored at 5 °C. Immediately after packaging and again after 10 days of storage, shrimp from each sub-batch were characterized by sensory evaluation (two packages), by microbiological and chemical methods (three packages), and by analysis of volatile compounds (three packages).

Sensory Analysis. Five or six trained panelists evaluated sensory changes by using a descriptive test. Shrimp samples were placed 30 min at 10 °C and then served in randomized order to the panelists. For evaluation of shrimps, after 0 and 10 days of storage, each panelist evaluated two portions of shrimp from each sub-batch (five or six shrimp per portion taken from the same package). Samples of freshly thawed shrimps were included in sensory sessions as controls. Changes in appearance, texture, odor, and flavor were characterized using descriptors of free choice, and the degree of overall changes was graded using a classical three class-scale with three corresponding to sensory rejection (*7*). Results are reported as average ± standard deviation (SD) for the two portions of shrimps from each sub-batch.

Microbiological and Chemical Analysis. Concentrations of aerobic plate counts (APC) were determined on Long and Hammer agar, lactic acid bacteria (LAB) in nitrite actidion polymyxin agar (NAP), and *B. thermosphacta* on STAA agar (*5*). NAP was used to allow growth of non-aciduric and acetate sensitive LAB including *Carnobacterium* spp. Dry matter, NaCl, pH, total volatile nitrogen (TVN), trimethylamine-oxide (TMAO), trimethylamine (TMA), and gas composition within packs were quantified by classical methods as previously described (*5*). Biogenic amines, organic acids, and free amino acids were detemined by using HPLC methods (*8*).

Analysis of Volatile Compounds. A dynamic headspace technique was used for extraction of volatile compounds from shrimp samples (*9*, *10*). In brief, shrimp were frozen in liquid nitrogen and homogenized to a fine powder. Sixty milligrams of tridecan (internal standard), 25 mL of distilled water, and antifoam were added to 5 g of this powder. Volatile compounds were then extracted at 45 °C with a flow of 340 mL of N/min and collected on 225 mg of Tenax GR (Chrompack, Bergen op Zoom, The Netherlands) in stainless steel tubes (Perkin-Elmer, Wellesley, MA). Following collection of volatile compounds, the tubes were purged with 50 mL of N/min to remove water vapor.

An automatic thermal desorber with a Tenax GR-packed cold-trap (ATD-400, Perkin-Elmer) was used to transfer volatiles from Tenax GR in stainless steel tubes to a 5890 IIA gas chromatograph (Hewlett-Packard, Palo Alto, CA). The GC was equipped with a DB 1701 column (30 m × 0.25 mm × 1.0 μm, J&W Scientific, Folsom, CA) and coupled to a HP 5972A mass selective detector. Helium was used as carrier gas with a flow of 1.3 mL/min. The temperature program was as follows: 35 °C held for 5 min, 2 °C/min to 45 °C, 4 °C/min to 165 °C, 30 °C/min to 240 °C, and finally 240 °C during 5 min. The GC-MS transfer line temperature was at 280 °C. The detector operated in a mass range between 30 and 250 with a scan rate at 3.35 scans/s (*9*, *10*). Identification of volatile compounds was carried out by using a MS database/library system (Wiley 138, Hewlett-Packard) and retention indices of known standard compounds from Sigma-Aldrich or Fluka. Data were reported as log(peak area/g) for each compound detected. The detection limit was ~220 000 area units corresponding to 4.6 log-(peak area/g).

Data Analysis. Different sensory characteristics and description of off-flavors of shrimp by each panelist after 10 days at 5 °C were scored as 1 (present) or 0 (absent), and principal component analysis (PCA) was used to evaluate differences between the nine sub-batches of shrimps. The possibility of predicting these sensory attributes (*Y* variables) from the chemical and microbiological characteristics (*X* variables) of shrimp was evaluated using partial least-squares regression (PLSR). Furthermore, the quantitative degree of shrimp spoilage, determined as the percentage of samples in class 3 (*Y* variable) was related to chemical and microbiological characteristics (*X* variables) by PLSR. Unscrambler (version 9.1, CAMO A/S, Trondheim, Norway) was used for multivariate data analysis. Prior to PLSR analyses, data for each variable were centered and scaled by subtracting the mean value and dividing by the SD across all samples. Full cross-validation of models was used. For each sampling time and sub-batch of shrimp the microbiological and chemical data were expressed as mean values ± SD ($n = 3$). The *t* test was used to evaluate differences between samples and treatments (Statgraphics Plus v. 5.1, Manugistics Inc., Rockville, MD).

RESULTS

***Carnobacterium* Species: Microbial, Sensory, and Chemical Changes during Storage of Shrimp.** The shrimp contained 1.3 ± 0.0% NaCl, 18.9 ± 0.3% dry matter, 80 ± 30 mg of N TVN/kg, 670 ± 140 mg of N TMAO/kg, and 614 ± 147 mg/kg lactic acid and had an initial pH of 7.6 ± 0.1. Prior to storage at 5 °C, the concentrations of APC, LAB, and *B. thermosphacta* were, respectively, ca. 3, 1, and <1 log(CFU/g). After 10 days at 5 °C, the concentration of these three groups of bacteria had increased to, respectively, 6.1, 4.8, and 4.5 log(CFU/g) in the noninoculated shrimp.

Table 2. Characteristics of Cooked and Peeled MAP Shrimp after Inoculation with *Carnobacterium* Species and 10 Days of Storage at 5 °C[a]

			C. maltaromaticum		
	noninoculated (control)	*C. divergens*	cluster H	cluster L	*C. mobile*
aerobic plate count [log(CFU/g)]	6.1 ± 0.2	9.4 ± 0.1	9.1 ± 0.2	9.4 ± 0.0	8.1 ± 0.1
lactic acid bacteria [log(CFU/g)]	4.8 ± 0.4	8.5 ± 0.3	9.0 ± 0.1	9.3 ± 0.1	8.1 ± 0.1
B. thermosphacta [log(CFU/g)]	4.6 ± 0.4	4.6 ± 0.4	4.9 ± 0.3	3.1 ± 0.9	4.6 ± 0.1
sensory characteristics	sea/seaweed weak sweet weak sour	chlorine chemical malty nutty sour nauseous/sweet	chlorine chemical malty nutty sour nauseous/sweet	grass/hay weak chlorine	yogurt-like
sensory rejection (% class 3)	13 ± 16	43 ± 20	43 ± 0[c,d]	8 ± 17	17 ± 24
free amino acids (mg/kg)					
arginine	2252 ± 201	8 ± 0[c]	428 ± 572[c]	10 ± 3[c]	2030 ± 122[d,e,g]
isoleucine	100 ± 9	98 ± 4	93 ± 4	93 ± 4	91 ± 5
leucine	199 ± 19	192 ± 11	187 ± 7	187 ± 10	175 ± 14
ornithine	245 ± 10	2033 ± 83[c]	1633 ± 402[c]	1947 ± 52[c]	333 ± 29[c,d,e,g]
tryptophan	39 ± 10	<5[c,d,f]	<5[c,d,f]	32 ± 5	30 ± 4[e,g]
tyrosine	81 ± 8	16 ± 4[c,d]	36 ± 7[c–e]	58 ± 6[c,e]	76 ± 4[d,e,g]
organic compounds (mg/kg)					
acetic acid, RRT 1.20[b]	<50[f]	391 ± 26[c]	374 ± 73[c]	393 ± 7[c]	< 50[d–g]
lactic acid, RRT 1.00	507 ± 89	655 ± 95	616 ± 90	475 ± 79	363 ± 62[c,e,g]
organic compounds (peak area)					
unidentified peak, RRT 0.88	174 ± 40	60 ± 58	64 ± 62	67 ± 11[c]	122 ± 26
unidentified peak, RRT 1.27	350 ± 128	ND[c,f]	27 ± 47[c]	ND[c,f]	266 ± 12[d,e,g]
unidentified peak, RRT 3.14	5097 ± 331	160 ± 277[c]	342 ± 593[c]	82 ± 142[c]	5071 ± 245[d,e,g]
tyramine (mg/kg)	<5[f]	60 ± 4[c,d]	42 ± 3[c–e]	20 ± 3[c]	<5[d–g]
total volatile nitrogen (mg of N/kg)	80 ± 30	440 ± 70[c]	370 ± 110[c]	470 ± 30[c]	110 ± 20[d,e]

[a] Values are averages ± standard deviation. $n = 3$ for microbiological and chemical analyses; $n = 2$ for sensory rejection except for noninoculated samples with $n = 4$. [b] Relative retention time (RRT) as compared to the retention time for lactic acid. [c] Indicates statistically significant difference of mean compared to noninoculated control ($P < 0.05$). [d] Indicates statistically significant difference of mean compared to cluster L ($P < 0.05$). [e] Indicates statistically significant difference of mean compared to *C. divergens* ($P < 0.05$). [f] Not detected due to signal below the limit of detection. [g] Indicates statistically significant difference of mean compared to *C. maltaromaticum* cluster H ($P < 0.05$)

To inoculated sub-batches of shrimp was added 4.5−5.5 log-(CFU/g) of the different mixtures of *Carnobacterium* isolates, and during storage at 5 °C growth to high concentrations was observed (**Table 2**). Growth and metabolism of *C. divergens* and *C. maltaromaticum* cluster H isolates in shrimp resulted in development of off-flavors and, particularly, in chlorine, malty, nutty, sour, and sweet off-flavors. For both sub-batches of shrimp, 43% of the samples were found to be sensorially unacceptable, that is, in class 3, after 10 days at 5 °C (**Table 2**). Shrimp inoculated with *C. maltaromaticum* cluster L or *C. mobile* isolates developed only very weak off-flavors, and these samples differed little from the noninoculated shrimps (**Figure 1**; **Table 2**).

The *Carnobacterium* species consumed arginine, tryptophan, tyrosine, and three unidentified organic compounds during growth in shrimp, with *C. mobile* being the least active (**Table 2**). The *C. divergens* and *C. maltaromaticum* cluster H isolates developed off-flavors of strongest intensity (corresponding to a higher percentage of rejected samples) and metabolized most tryptophan and tyrosine. These isolates also produced the highest concentrations of tyramine (**Table 2**), 2-ethyl-1-hexanol, 3-heptanol, 1-octen-3-ol, 1-pentanol, 2,3-butanedione (diacetyl), 3-heptanone, 2-heptanone, 2-hexanone, 4-methyl-2-pentanone, 2,4-pentanedione, 2-pentanone, 3-pentanone, 4-methyl-3-penten-2-one, 2,4,6-trimethylpyridine, and five unidentified volatile compounds (**Table 4**). *C. maltaromaticum* cluster L and *C. mobile* developed off-flavors of weakest intensity but, compared to *C. divergens* and *C. maltaromaticum* cluster H, *C. maltaromaticum* cluster L produced as much or more of the following metabolites: ornithine, acetic acid, total volatile nitrogen/ammonia (**Table 2**), 3-methyl-1-butanal, and 3-methyl-1-butanol (**Table 4**).

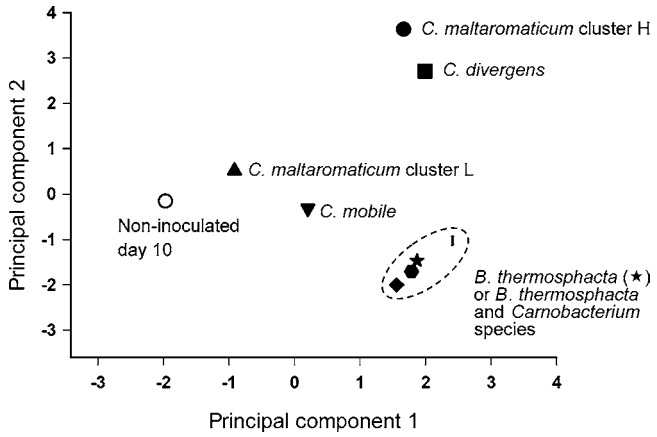

Figure 1. Scores from PCA of sensory attributes of inoculated and noninoculated shrimp. All samples were stored for 10 days at 5 °C. PC 1 and PC 2 explained 30 and 21%, respectively, of the variation in sensory attribute data. PC 1 accounted for variation in the attributes buttermilk-like and nauseous/sweet (positive correlation) as well as ocean/seaweed (negative correlation). PC 2 accounted for variation in chlorine and chemical flavor (positive correlation) as well as butter and wet dog (negative correlation).

The growth and activity of the *Carnobacterium* species slightly reduced ($P < 0.05$) the concentration of headspace O_2 from 28.9 to 23.5−25.5%. The effect on headspace CO_2 (31.4−36.2%) and pH (7.4−7.7) of the shrimp was little and not significant for all sub-batches, and this was also the case for the following free amino acids in shrimp meat (mg/kg): alanine (588 ± 69), aspartic acid (14 ± 2), glutamic acid (168 ± 21),

Table 3. Characteristics of Cooked and Peeled MAP Shrimp after Inoculation with *Carnobacterium* Species and/or *B. thermosphacta* and 10 Days of Storage at 5 °C[a]

		B. thermosphacta and *Carnobacterium* species		
	B. thermosphacta	*C. divergens*	*C. maltaromaticum*, cluster H	*C. mobile*
aerobic plate count [log(CFU/g)]	8.3 ± 0.2	9.1 ± 0.2	8.8 ± 0.1	8.3 ± 0.3
lactic acid bacteria [log(CFU/g)]	6.6 ± 0.2	8.4 ± 0.4	8.1 ± 0.3	7.7 ± 0.3
B. thermosphacta [log (CFU/g)]	8.3 ± 0.2	8.3 ± 0.4	8.3 ± 0.1	8.2 ± 0.3
sensory characteristics	strong butter buttermilk sour nauseous sweet	butter wet dog metal nauseous sweet ammonia	butter wet dog metal pungent	strong butter buttermilk wet dog nauseous sweet dishcloth
sensory rejection (% class 3)	83 ± 24[c]	67 ± 0[c]	75 ± 12[c]	83 ± 0[c]
free amino acids (mg/kg)				
arginine	2050 ± 170	937 ± 902	1720 ± 228[c,f]	2149 ± 144
isoleucine	58 ± 5[c]	55 ± 28	55 ± 13[c,f]	70 ± 8[c]
leucine	120 ± 14[c]	142 ± 33	136 ± 17[c,f]	150 ± 27[c]
ornithine	236 ± 9	1284 ± 798	880 ± 207[f]	275 ± 10[c,d]
tryptophan	30 ± 2	33 ± 3	34 ± 2[f]	33 ± 3
tyrosine	74 ± 8	41 ± 32	46 ± 1[c,d]	82 ± 1
organic compounds (mg/kg)				
acetic acid, RRT[b] 1.20	<50[g]	401 ± 68[c,d]	340 ± 9[c,d]	<50[g]
lactic acid, RRT 1.00	484 ± 39	550 ± 44	550 ± 25	381 ± 95
organic compounds (peak area)				
unidentified peak, RRT 0.88	ND[c,g]	77 ± 15[c,d]	23 ± 40[c]	ND[c,g]
unidentified peak, RRT 1.27	143 ± 24[c]	ND[c,d,g]	32 ± 71[c]	129 ± 82[c]
unidentified peak, RRT 3.14	5470 ± 278	81 ± 80[c,d]	1973 ± 1346[c,d]	4733 ± 404
tyramine (mg/kg)	<5[g]	52 ± 3[c,d]	29 ± 3[c,d,f]	<5[g]
total volatile nitrogen (mg of N/kg)	100 ± 20	550 ± 120[c,d]	300 ± 60[c,d]	120 ± 0[c]

[a] Values are averages ± standard deviation. $n = 3$ for microbiological and chemical analyses; $n = 2$ for sensory rejection. [b] Relative retention time (RRT) as compared to the retention time for lactic acid. [c] Indicates statistically significant difference of mean compared to noninoculated control ($P < 0.05$). [d] Indicates statistically significant difference of mean compared to shrimp inoculated with *B. thermosphacta* alone ($P < 0.05$). [e] Indicates statistically significant difference of mean compared to shrimp inoculated with *C. divergens* alone ($P < 0.05$). [f] Indicates statistically significant difference of mean compared to shrimp inoculated with *C. maltaromaticum* alone ($P < 0.05$). [g] Not detected due to signal below the limit of detection.

glycine (3860 ± 302), histidine (122 ± 24), isoleucine (109 ± 15), leucine (216 ± 32), lysine (221 ± 23), methionine (158 ± 27), phenylalanine (106 ± 17), proline (1359 ± 291), serine (223 ± 21), threonine (400 ± 61), and valine (172 ± 25).

***B. thermosphacta* and *Carnobacterium* Species: Microbial, Sensory, and Chemical Changes during Storage of Shrimp.** Shrimp with microbiological and chemical characteristics as reported above were inoculated with 4.5−5.0 log(CFU/g) of *B. thermosphacta* and 4.8−5.5 log(CFU/g) of the different *Carnobacterium* species. After inoculation, both *B. thermosphacta* and *Carnobacterium* species grew to high concentrations after 10 days at 5 °C (**Table 3**). Inoculated sub-batches of shrimp all developed off-flavors of strong intensity, but spoilage characteristics differed markedly between shrimps inoculated with *B. thermosphacta* alone (strong butter/buttermilk-like and sour) and shrimp inoculated with cocultures of *B. thermosphacta* and *C. divergens* or *C. maltaromaticum* (butter and wet dog). *C. mobile*, however, had little effect on off-flavors and metabolites produced by *B. thermosphacta* in shrimp (**Tables 3** and **4**).

B. thermosphacta consumed isoleucine, leucine, and three unidentified organic compounds during growth in shrimp and produced 2,3-butanedione (diacetyl), 3-methyl-1-butanal, 2-octene, 3-methyl-1-butanol, dimethylbenzene, and butanediol as well as an unidentified compound with a GC-MS retention index of 1079 (**Tables 3** and **4**). Of these volatile compounds 2,3-butanedione (diacetyl), 3-methyl-1-butanal, 3-methyl-1-butanol, and the unidentified volatile compound (RI = 1079) were indicated by GC−olfactometry to be odor active (results not shown), and these compounds seem to be important in explaining off-odor formation by *B. thermosphacta* in chilled MAP shrimp (**Table 3**). Cocultures of *B. thermosphacta* and *C. divergens* or *C. maltaromaticum* cluster H formed less 2,3-butanedione and 3-methyl-1-butanal and more acetic acid and ammonia (total volatile nitrogen) than *B. thermosphacta* when growing alone in shrimp (**Tables 3** and **4**). When growing alone or in cocultures with *Carnobacterium* species, *B. thermosphacta* reduced ($P < 0.05$) the concentration of headspace O_2 to 23.5−24.4% and increased ($P < 0.05$) the concentration of headspace CO_2 from 31.4 ± 3.0 to 36.0−38.6%. The effect on the pH (7.2−7.5) of the shrimps was limited and not significant for all sub-batches, and this was also the case for concentrations of the free amino acids alanine, aspartic acid, glutamic acid, glycine, histidine, lysine, methionine, phenylalanine, proline, serine, threonine, and valine.

Multivariate Data Analysis: Sensory, Chemical, and Microbial Changes during Storage of Shrimp. The sensory rejection of shrimps determined as the percentage of samples in class 3 (*Y* variable) was predicted from chemical and microbiological changes. A model with four PLS components explained 89% of the variation in the *Y* variable (**Figure 2**). The relationship between explained variance of the *Y* variable and the number of PLS components in models indicated the four-PLS-component model as appropriate. This PLSR model explained 69% of the variation in the chemical and microbiological data, indicating that a subset of the 61 *X* variables could be sufficient to predict the percentage of samples in class 3. In fact, a less complex PLSR model with one PLS component and four *X* variables (diacetyl, isoleucine, tyramine, and an unidentified organic compound with HPLC relative retention time of 0.88) explained 84% of the variation in the *Y* variable. Furthermore, a one-PLS-component model with only three *X* variables (diacetyl, isoleucine, and tyramine) explained 78% of the variation in the *Y* variable. Values of the regression

Table 4. Development of Volatile Compounds in Cooked and Peeled MAP Shrimp after Inoculation with *Carnobacterium* Species and/or *B. thermosphacta* Followed by Storage during 10 Days at 5 °C[a]

volatile compound	RI[b]	ID[c]	non-inoculated (control)	*C. divergens*	*C. maltaromaticum* cluster H	*C. maltaromaticum* cluster L	*C. mobile*	*B. thermosphacta*	*B. thermosphacta* and *C. divergens*	*B. thermosphacta* and *C. maltaromaticum*, cluster H	*B. thermosphacta* and *C. mobile*
alcohols											
butanediol	943	B	5.3 ± 0.8	<4.6[e]	<4.6[e]	6.1 ± 0.0	6.0 ± 0.1	6.2 ± 0.0[d,f,h]	6.1 ± 0.1[f]	6.2 ± 0.1[d,h]	6.1 ± 0.0[g]
2-ethyl-1-hexanol	1127	B	5.4 ± 0.8	6.2 ± 0.0[e]	6.2 ± 0.1[e]	<4.6	<4.6	<4.6[f,h]	<4.6[f]	<4.6[h]	<4.6
3-hexanol	890	B	<4.6	5.5 ± 0.7	<4.6	<4.6	<4.6	<4.6	<4.6	<4.6	<4.6
2-hexanol	897	B	<4.6	5.6 ± 0.9	5.1 ± 0.8	<4.6	<4.6	<4.6	<4.6	<4.6	<4.6
3-heptanol	986	B	6.3 ± 0.2	6.6 ± 0.0[d,e]	6.6 ± 0.0[d,e]	6.1 ± 0.1	5.1 ±0.8	6.2 ± 0.1[f,h]	6.1 ± 0.0[f]	5.6 ± 0.9	5.6 ± 0.8
3-methyl-1-butanol	847	A	6.2 ± 0.2	7.4±0.1[d,e]	7.9 ± 0.0[d,f]	7.9±0.1[d]	<4.6[d,e]	7.2 ± 0.1[d,f,h]	6.9 ± 0.0[d,f,g]	7.0 ± 0.1[d,h]	7.2 ± 0.1[d]
2-methyl-1-butanol	849	A	<4.6	6.7±0.1[d,e]	7.1 ± 0.0[d,f]	7.2±0.1[d]	<4.6[e]	6.6 ± 0.1[d,f,h]	<4.6[f,g]	5.6 ± 0.8	6.7 ± 0.3[d]
1-octen-3-ol	1070	B	5.4 ± 0.9	6.2 ± 0.0[e]	6.2 ± 0.0[e]	<4.6	<4.6	6.2 ± 0.1[f,h]	6.1 ± 0.0[f]	6.0 ± 0.2[h]	5.6 ± 0.9
1-pentanol	877	B	5.4 ± 0.9	6.2 ± 0.0[e]	6.2 ± 0.0[e]	<4.6	<4.6	<4.6[f,h]	<4.6[f]	<4.6[h]	<4.6
1-penten-3-ol	790	A	5.5 ± 0.7	5.5 ± 0.8	<4.6[d,e]	6.3 ± 0.1[d]	6.0 ± 0.1[e]	6.2 ± 0.1[d,h]	6.1 ± 0.0[d]	6.0 ± 0.2[h]	5.6 ± 0.9
aldehydes											
3-methyl-1-butanal	737	A	<4.6	6.7±0.1[d,e]	7.2±0.1[d,f]	7.2±0.0[d]	<4.6[e]	6.9 ± 0.1[d,f,h]	6.5 ± 0.1[d,f,g]	6.4 ± 0.1[d,g,h]	6.8 ± 0.1[d]
2-methyl-1-butanal	743	B	<4.6	5.6 ±0.8	6.6±0.1[d]	6.5±0.0[d]	<4,6[e]	6.5 ± 0.1[d]	<4.6[g]	<4.6[g,h]	6.5 ± 0.1[d]
ketones											
2,3-butanedione	684	A	5.6 ± 1.1	6.7 ± 0.0[e]	6.7 ± 0.1[e]	6.1 ± 0.1	6.5 ± 0.1[e]	7.3 ± 0.1[d,f,h]	5.6 ± 0.9	6.1 ± 0.1[g,h]	7.3 ± 0.2[d]
3-heptanone	970	A	5.6 ± 1.1	6.7 ± 0.0[e]	6.7 ± 0.0[e]	<4.6	<4.6	<4.6[f,h]	<4.6[f]	<4.6[h]	<4.6
2-heptanone	980	A	5.4 ± 0.9	6.2 ± 0.0[e]	6.2 ± 0.0[e]	<4.6	<4.6	<4.6[f,h]	<4.6[f]	<4.6[h]	<4.6
2-hexanone	880	A	5.7 ± 1.2	6.8 ± 0.0[e]	6.8 ± 0.0[e]	<4.6	<4.6	<4.6[f,h]	<4.6[f]	<4.6[h]	<4.6
4-methyl-2-pentanone	826	A	5.5 ± 1.0	6.4 ± 0.1[e]	6.3 ± 0.0[e]	<4.6	<4.6	<4.6[f,h]	<4.6[f]	<4.6[h]	<4.6
2,4-pentanedione	869	B	5.8 ± 1.3	7.0 ± 0.1[e]	6.9 ± 0.0[e]	<4.6	<4.6	<4.6[f,h]	<4.6[f]	<4.6[h]	<4.6
2-pentanone	777	A	5.8 ± 1.3	7.0 ± 0.1[e]	6.8 ± 0.1[e,f]	<4,6	<4.6	<4.6[f,h]	<4.6[f]	<4.6[h]	<4.6
3-pentanone	783	B	5.5 ± 1.0	6.5 ± 0.1[e]	6.3 ± 0.0[e,f]	<4.6	<4.6	<4.6[f,h]	<4.6[f]	<4.6[h]	<4.6
4-methyl-3-penten-2-one	884	B	5.7 ± 1.2	6.9 ± 0.0[e]	6.8 ± 0.0[e]	<4.6	<4.6	<4.6[f,h]	<4.6[f]	<4.6[h]	<4.6
hydrocarbons											
dimethylbenzene	921	B	<4.6	<4.6	<4.6	<4.6	<4.6	6.8 ± 0.1[d,f,h]	5.1 ± 0.8	6.0 ± 0.0[d,g,h]	6.6 ± 0.0[d]
2-octene	815	B	5.5 ± 1.0	<4.6	<4.6	<4.6	<4.6	6.2 ± 0.4[f,h]	6.3 ± 0.1[f]	5.7 ± 0.9	<4.6[g]
miscellaneous											
2,4,6-trimethylpyridine	1061	B	<4.6	6.1 ± 0.1[d,e]	6.5 ± 0.1[d,e,f]	<4.6	<4.6	<4.6[f,h]	<4.6[f]	<4.6[h]	<4.6
unidentified peak	1041		6.3 ± 0.1	6.3 ± 0.0[e]	6.3 ± 0.0[e]	6.5 ± 0.0[d]	6.4 ± 0.0[d]	6.4 ± 0.0[f,h]	6.6 ± 0.0[d,f,g]	6.6 ± 0.1[d,g,h]	6.5 ± 0.0[d]
unidentified peak	1079		5.6 ± 1.0	<4.6[e]	<4.6[e]	6.5 ± 0.1	6.4 ± 0.1	6.5 ± 0.1[f,h]	6.5 ± 0.1[f]	6.8 ± 0.2[d,h]	6.4 ± 0.1
unidentified peak	1135		5.4 ± 0.9	6.3 ± 0.0[e]	6.3 ± 0.0[e]	<4.6	<4.6	<4.6[f,h]	6.0 ± 0.0[f,g]	5.6 ± 0.8	<4.6
unidentified peak	1164		5.7 ± 1.2	6.8 ± 0.0[e]	6.8 ± 0.0[e]	<4.6	<4.6	<4.6[f,h]	<4.6[f]	<4.6[h]	<4.6
unidentified peak	1197		5.6 ± 1.1	6.6 ± 0.0[e]	6.7 ± 0.0[e,f]	<4.6	<46	<4.6[f,h]	<4.6[f]	<4.6[h]	<4.6

[a] Values are averages ± standard deviation of log (peak area/g). $n = 3$. [b] Retention (Kovat's) index on DB-1701 capillary column. [c] A, identification on the basis of MS database/library system and retention index of reference compounds from Sigma-Aldrich or Fluka; B, identification on the basis of MS database/library system. [d] Indicates statistically significant difference of mean compared to noninoculated control ($P < 0.05$). [e] Indicates statistically significant difference of mean compared to cluster L alone ($P < 0.05$). [f] Indicates statistically significant difference of mean compared to *C. divergens* alone ($P < 0.05$). [g] Indicates statistically significant difference of mean compared to shrimp inoculated with *B. thermosphacta* alone ($P < 0.05$). [h] Indicates statistically significant difference of mean compared to shrimps inoculated with *C. maltaromaticum* cluster H alone ($P < 0.05$).

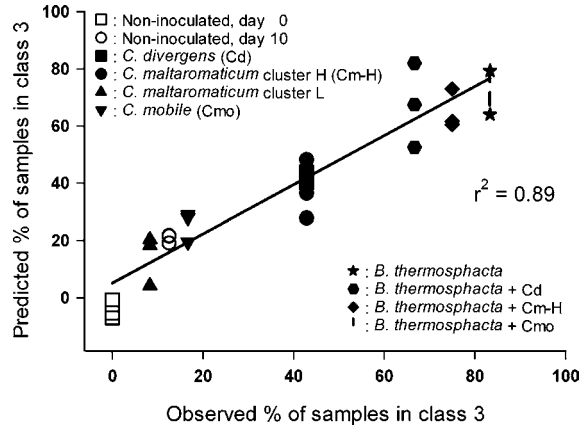

Figure 2. Correlation between the observed average percentage of sensory rejected shrimp samples (samples in class 3) and values of this parameter predicted by a PLSR model relying on 61 chemical and microbiological characteristics measured in triplicate of the shrimp samples. Shrimp samples were analyzed by sensory, chemical, and microbiological methods after 10 days of storage at 5 °C.

coefficients in the original four-PLS-component model were used to select *X* variables for less complex models. As expected, the *X* variables retained in simple PLS models corresponded to substrates or metabolites of *B. thermosphacta* (isoleucine), *Carnobacterium* species (tyramine), or both groups of bacteria (diacetyl and the unidentified organic compound with HPLC relative retention time of 0.88).

Chemical and microbial characteristics of shrimp were related to their off-flavors, but a PLSR model with three PLS components explained only 52% of the variation in sensory descriptors for all sub-batches of shrimp when analyzed together. The sensory descriptors were more appropriately predicted when data for shrimps inoculated with *Carnobacterium* species, *B. thermosphacta*, or *Carnobacterium* species in coculture with *B. thermosphacta* were analyzed separately, but five to eight PLS components were required to explain 77−90% of the variance in sensory characteristics for the three data sets.

DISCUSSION

The growth and activity of *C. divergens* and *C. maltaromaticum* cluster H resulted in similar and equally intensive off-flavors in shrimps (**Figures 1** and **2**; **Table 2**). For *C. divergens*, off-odor formation was also previously observed in chilled vacuum-packed beef (*11*). For *C. maltaromaticum* our results confirmed its spoilage potential in cooked and peeled MAP shrimp (*5*). *C. maltaromaticum* is known to form malt-like flavors in milk and to change the sensory characteristics of beef

and cooked ham (*12*−*14*), but it may contribute positively to the aroma of soft cheeses and sausages (*15*, *16*). However, *C. maltaromaticum* cluster L showed less spoilage activity than *C. maltaromaticum* cluster H (**Figures 1** and **2**), and this is interesting as preliminary antibacterial screenings (*1*) indicated *C. maltaromaticum* cluster L may have potential as a protective culture.

In agreement with their sensory spoilage potential, the metabolic activities of *C. divergens* and *C. maltaromaticum* cluster H isolates in shrimp meat were similar, although *C. divergens* formed 2-hexanol and 3-hexanol and *C. maltaromaticum* formed relatively higher concentrations of 2- and 3-methyl-1-butanal and 2- and 3-methyl-1-butanol (**Figure 1**; **Table 4**). In laboratory media *C. divergens* and *C. maltaromaticum* produce acetate, acetoin, CO_2, ethanol, formate, and lactate from glucose; ornithine and ammonia from arginine; tyramine from tyrosine; α-ketoisocaproate, 3-methyl-1-butanal and 3-methyl-1-butanol from leucine; 2-methyl-1-butanal and 2-methyl-1-butanol from isoleucine; and 2-methyl-1-propanal and 2-methyl-1-propanol from valine (*17*−*21*). Using the API-ZYM system *C. maltaromaticum* was also shown to display esterase activity (*15*). In cold-smoked salmon (40 days, 6 °C) *C. maltaromaticum* isolates, together with other metabolites, formed 2,3-butanedione (diacetyl) and 2,3-pentanedione, but this product was not considered to be spoiled by the studied isolates (*6*) or by isolates of *C. divergens* (*22*). In chilled milk and cooked ham *C. maltaromaticum* formed 3-methyl-1-butanal, 3-methyl-1-butanol, 2-methyl-1-propanal, and 2-methyl-1-propanol, and the aldehydes have been suggested to convey the malty aroma that is characteristic of *C. maltaromaticum* grown in milk (*12*, *14*, *21*, *23*). In cooked MAP shrimp *C. maltaromaticum* cluster H, *C. maltaromaticum* cluster L, and *C. divergens* formed 3-methyl-1-butanal and 3-methyl-1-butanol but not detectable amounts of 2-methyl-1-propanal or 2-methyl-1-propanol, although the substrate valine was available. In a sausage mince a *C. maltaromaticum* isolate consumed leucine and formed acetoin, α-ketoisocaproate, and 3-methylbutanoic acid, but 3-methyl-1-butanal and 3-methyl-1-butanol were not detected, although the isolate formed these metabolites from leucine when grown in a liquid medium (*16*). This corresponds to the inability of *C. maltaromaticum* and *C. divergens* to form 2-methyl-1-propanal or 2-methyl-1-propanol in cooked MAP shrimp. Specific product characteristics of the cooked shrimps (e.g., a high pH value) may influence their metabolic activity, but further studies are required to evaluate this assumption. The limited formation of acetoin, CO_2, formate, and lactate by carnobacteria probably resulted from a low glucose/carbohydrate concentration, which is typical for these shrimps (*P. borealis*). In shrimp, *C. divergens* and *C. maltaromaticum* cluster H isolates formed acetic acid, but the ∼400 mg/kg (0.04%) produced is unlikely to influence sensory characteristics as confirmed by the same concentration being produced by the *C. maltaromaticum* cluster L isolates, although they did not form spoilage off-flavors (**Table 2**).

As discussed above, 3-methyl-1-butanal and 3-methyl-1-butanol have been associated with a malty odor (*12*). The malty off-flavors produced by *C. divergens* and *C. maltaromaticum* cluster H (**Table 2**) could, however, not solely result from 3-methyl-1-butanal and/or 3-methyl-1-butanol as *C. maltaromaticum* cluster L isolates formed similar concentrations of these compounds but no malty off-flavors (**Table 2**).

Several ketones with five or more carbon atoms were formed by *C. divergens* and *C. maltaromaticum* cluster H but not by *C. maltaromaticum* cluster L or *C. mobile* (**Table 4**). These ketones are odor active with characteristics including chemical, fruity, musty, soapy, and sweet (*24*−*26*). They may contribute to spoilage off-flavors formed by some carnobacteria, and this deserves further study. Esterase activity of *C. maltaromaticum* was previously observed (*15*), and the ketones may result from the lipid content of ∼1.0% in cooked and peeled shrimp (*P. borealis*). Furthermore, *C. divergens* and *C. maltaromaticum* cluster H consumed tryptophan (**Table 2**), and this metabolic activity may also be related to off-odor formation. The isolates studied, however, were all unable to form indole in vitro and did not produce tryptamine or serotonine (results not shown) and we could not determine how tryptophan was metabolized.

The observed formation of ornithine and ammonia from arginine and of tyramine from tyrosine was expected for *C. divergens* and *C. maltaromaticum*, but, interestingly, *C. maltaromaticum* cluster L isolates formed significantly ($P < 0.001$) less tyramine (**Table 2**). The oral toxicity of tyramine is low, and in seafood the concentrations produced by carnobacteria will have no adverse effects on most consumers. However, for sensitive individuals, for example, with reduced monoamine oxidase activity due to medication or hereditary deficiency, very little tyramine can cause migraine headaches, and intake of no more than 5 mg of tyramine per meal has been recommended (*27*−*29*). In cold-smoked salmon *C. divergens* and *C. maltaromaticum* formed ∼300 mg/kg of tyramine during storage at 4−8 °C (*22*, *30*, *31*), whereas *C. maltaromaticum* formed 40−60 mg/kg tyramine in frozen and thawed MAP salmon at 2 °C (*3*). Although the use of classical monoamine oxidase inhibitors as antidepressants is decreasing, high concentrations of tyramine in seafood remain critical for some individuals. In fact, it has been suggested that a *C. divergens* isolate in which the tyrosine decarboxylase gene was inactivated by mutagenesis be used as a protective culture to prevent growth of *L. monocytogenes* in cold-smoked salmon (*32*).

The present study confirmed the observation of Mejlholm et al. (*5*) that a coculture of *B. thermosphacta* and *C. maltaromaticum* form a particular wet dog off-flavor in shrimp, although mixtures of *B. thermosphacta* isolates or mixtures of *C. maltaromaticum* isolates, when studied separately, did not form this off-flavor (**Tables 2** and **3**). As compared to mixtures of *B. thermosphacta* or *C. maltaromaticum* isolates we found no new metabolites produced by cocultures of *B. thermosphacta* and *C. maltaromaticum* (**Tables 2**−**4**). Thus, the wet dog off-flavor may not result from metabiosis, that is, where one bacteria uses a product of another bacteria as substrate to form one or several new metabolites. On the contrary, *C. maltaromaticum* markedly reduced the diacetyl formation of *B. thermosphacta*, and *B. thermosphacta* reduced the activity of *C. maltaromaticum* as shown from the consumption of amino acids and formation of various metabolites (**Tables 2**−**4**). Thus, the wet dog off-flavor may result from interaction between metabolites formed by *C. divergens*, *C. maltaromaticum* cluster H, and to some extent *C. mobile* and those produced by *B. thermosphacta* (**Tables 3** and **4**).

Oxygen availability increases the spoilage potential and the number of metabolites produced by *B. thermosphacta*. For modified atmospheres, concentrations of both O_2 and CO_2 determine if metabolism of *B. thermosphacta* is primarily anaerobic or aerobic (*33*−*35*). According to the model suggested by Pin et al. (*34*), the concentration of O_2 required for the metabolism of *B. thermosphacta* to be aerobic increases with the concentration of CO_2. With 33.5−38.6% CO_2 and 23.5−24.5% O_2, as determined in the present study, the metabolism of *B. thermosphacta* should be anaerobic, and with this level

of CO_2 >32% O_2 would be needed for the metabolism of *B. thermosphacta* to become primarily aerobic (*34*). However, in the cooked and peeled MAP shrimp, *B. thermosphacta* formed butanediol and 2,3-butanedione (**Table 4**), indicating its metabolism to be at least partly aerobic (*33*). To reduce the spoilage potential of *B. thermosphacta* in cooked and peeled MAP shrimp, an atmosphere with less O_2 and more CO_2 may be used. However, to extend the shelf life of this product, the potential for growth of *L. monocytogenes* and not only the metabolism of *B. thermosphacta* must be limited (*5*).

As shown in the present study *C. maltaromaticum* cluster L isolates had limited spoilage activity and formed less tyramine than other studied isolates of *C. maltaromaticum* and *C. divergens* (**Table 2**). When used as protective culture in high concentration in cooked and peeled MAP shrimp, *C. maltaromaticum* cluster L, unfortunately, was (i) unable to prevent growth of *L. monocytogenes*, (ii) unable to markedly reduce growth of *B. thermosphacta*, and (iii) unable to clearly delay sensory spoilage of the product (results not shown). Further studies are required to determine if *C. maltaromaticum* cluster L isolates may be applicable as protective cultures in lightly preserved foods where their antimicrobial properties will act in combination with product characteristics such as reduced pH and increased NaCl concentrations.

The present study has provided new information concerning the metabolic activity of *Carnobacterium* species in shrimp and contributed to an understanding of their role as spoilage bacteria and potential protective cultures. It seems relevant to study the metabolic and spoilage activity of *Carnobacterium* species in other products and to further evaluate their potential and limitations as protective cultures.

ACKNOWLEDGMENT

We thank Carina Vigsø Christensen, Tina Julien, and Nadereh Samieian from DIFRES for their technical assistance. Anne-Mette Haahr from DIFRES helped with analysis of volatile compounds.

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Received for review December 2, 2005. Revised manuscript received March 11, 2006. Accepted March 19, 2006. The study was financed by the Danish Agricultural and Veterinary Research Council (Project 23-02-0142).

JF053017F