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Significance of Heme-Based Respiration in Meat Spoilage Caused by *Leuconostoc gasicomitatum*

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***Leuconostoc gasicomitatum* is a psychrotrophic lactic acid bacterium (LAB) which causes spoilage in cold-stored modified-atmosphere-packaged (MAP) meat products. In addition to the fermentative metabolism, *L. gasicomitatum* is able to respire when exogenous heme and oxygen are available. In this study, we investigated the respiration effects on growth rate, biomass, gene expression, and volatile organic compound (VOC) production in laboratory media and pork loin. The meat samples were evaluated by a sensory panel every second or third day for 29 days. We observed that functional respiration increased the growth (rate and yield) of *L. gasicomitatum* in laboratory media with added heme and *in situ* meat with endogenous heme. Respiration increased enormously (up to 2,600-fold) the accumulation of acetoin and diacetyl, which are buttery off-odor compounds in meat. Our transcriptome analyses showed that the gene expression patterns were quite similar, irrespective of whether respiration was turned off by excluding heme from the medium or mutating the *cydB* gene, which is essential in the respiratory chain. The respiration-based growth of *L. gasicomitatum* in meat was obtained in terms of population development and subsequent development of sensory characteristics. Respiration is thus a key factor explaining why *L. gasicomitatum* is so well adapted in high-oxygen packed meat.**

Leuconostoc gasicomitatum was described and associated with food spoilage for the first time in 1996 (1). Since then, it has been implicated in causing spoilage in many types of cold-stored modified-atmosphere-packaged (MAP) foods of several manufacturers in Finland (2, 3). The pathways/genes associated with many types of spoilage reactions, such as a buttery off-odor, gas production, meat greening, and slime formation, have been identified through the annotation process (4), and the fully assembled genome of *L. gasicomitatum* LMG18811^T has been made available (GenBank accession no. FN822744). *L. gasicomitatum* spoilage has mainly affected marinated broiler meat (5, 6) or high-oxygen MAP beef (2, 7, 8). Marinating has been shown to be a predisposing factor for *L. gasicomitatum* spoilage (8), and the presence of oxygen in the packaging atmosphere has been suspected to be another factor favoring growth of *L. gasicomitatum* in MAP meat.

The members of the genus *Leuconostoc* are obligatory heterofermentative lactic acid bacteria (LAB) that produce lactic acid, ethanol, acetic acid, and CO_2 from carbohydrates. In addition to the fermentative metabolism, some LAB are able to respire when exogenous heme is available (9–13). *L. gasicomitatum* type strain LMG18811 belongs to this subgroup of LAB (4). Respiration requires three main membrane components: an NADH dehydrogenase (electron donor), a quinone (which delivers electrons), and cytochrome oxidase (electron acceptor protein). All respiring LAB encode a single cytochrome *bd* oxidase, the activity of which has been shown to be heme dependent (13, 14).

Respiration has been shown to result in increased biomass, long-term survival, and several changes in the metabolism of LAB (13, 15, 16). Despite the documentation of the respiration capacity of several LAB in laboratory media, there is no knowledge of whether meat spoilage LAB, such as *L. gasicomitatum*, are able to benefit from the ability to respire in chilled, high-oxygen MAP meat. This is the first study of the consequences of LAB respiration in meat, which naturally contains heme. We show that heme-based respiration significantly changes the metabolism and increases the growth of *L. gasicomitatum* in high-oxygen MAP meat.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. The bacterial strains used in this study are shown in Table 1. Unless otherwise stated, bacteria were cultivated at 25°C in de Man-Rogosa-Sharpe medium (MRS; Oxoid, Basingstoke, United Kingdom). Broth cultures were cultivated without shaking. MRS plates were incubated in jars made anaerobic by a commercial atmosphere generation system (Anaerogen; Oxoid, Basingstoke, United Kingdom).

Mutagenesis for making a recombinant strain without a functional electron transport chain. Plasmid pSIP256 was created by cutting the plasmid pSIP409 (17) with SalI and XhoI and religation. An 870-bp internal cytochrome *d* ubiquinol oxidase, subunit II (*cydB*, LEGAS_1333), gene fragment was amplified by PCR from *L. gasicomitatum* LMG18811^T, using the primers cydB_F05 (aatgaattcTAATGCGCGTGATCAAAAAG) and cydB_R04 (ttaaaagcttATAACCGAGCGTGATGGGTA), where the lowercase nucleotides represent restriction sites. The PCR fragment was cleaved with EcoRI and HindIII and cloned into the plasmid pSIP256 to generate the plasmid pSIP1333A. Plasmid pSIP1333A was used to transform *L. gasicomitatum* LMG18811^T, essentially according to the method of Helmark et al. (18), selecting for erythromycin (3 μg ml^{-1}) resistance. Transformants were checked by PCR using external PCR primers cydA2

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TABLE 1 Strains used in this study

L. gasicomitatum strain	Origin	Reference or source
LMG18811^T	Spoiled MAP marinated broiler strips	1
LMG18811^T::pSIP1333A	*cydB* single-crossover mutant of LMG18811^T	This paper
RSNS1b	Spoiled MAP beef	7
N20-1	MAP unmarinated broiler strips	5
HHSL3c	MAP marinated broiler strips	5
ITTJA1a	MAP marinated broiler strips	7
POK13-6	MAP pork strips	7
NAPA6-6	MAP beef strips	7
NAGRM3a-2	Vacuum-packaged beef	7
NALP2-7	Beef	7
SISU-4-3	MAP pork strips	7
RSNL2b	Beef	7

(GGTGGTGTGATGGTTTGGTT) and cydC2 (ACCGCCTTGTCAGCA TAAAC). The recombinant strain was designated *L. gasicomitatum* LMG18811::pSIP1333A, *cydB* mutant (Table 1).

Respiration potential of *L. gasicomitatum* strains in broth culture. The effect of heme-based respiration on the growth of the *L. gasicomitatum* strains (Table 1) was investigated by cultivating the bacteria in a Bioscreen C microbiological reader (Oy Growth Curves AB, Ltd., Helsinki, Finland). Microtiter plates, specifically manufactured for this device, were loaded with 400 μl of MRS broth (Oxoid, Basingstoke, United Kingdom) with or without 2 μg ml^{-1} heme (hemin; Sigma-Aldrich) in dimethyl sulfoxide (DMSO). In the medium without heme, an equivalent amount (2 μl ml^{-1}) of DMSO was added. The cell density at the beginning of the experiment was approximately 10^4 exponential-phase CFU ml^{-1}. Bacteria were cultivated under continuous shaking (low speed) at 6°C for 12 days either under air or in an anaerobic cabinet (MK III; Don Whitley Scientific, Ltd., Shipley, United Kingdom) containing 85% N_2, 10% CO_2, and 5% H_2. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) at 60-min intervals. Two separate Bioscreen runs with three biological replicates were made, and those six replicates were used for calculating the standard deviation.

Microarrays and transcriptome analyses. LMG18811^T (wild type) and LMG18811^T::pSIP1333A (*cydB* mutant) were grown at 25°C and 20 rpm in MRS broth with or without 2 μg ml^{-1} heme until the OD_{600} value was 0.6 (Biophotometer; Eppendorf, Germany), corresponding to approximately 2×10^8 CFU ml^{-1}. Two or three independent replicates were done. The following transcriptomes were compared: (i) wild type cultivated with heme versus wild type cultivated without heme, (ii) wild type cultivated with heme versus *cydB* mutant cultivated with heme, (iii) wild type cultivated with heme versus *cydB* mutant cultivated without heme, and (iv) *cydB* mutant cultivated with heme versus *cydB* mutant cultivated without heme. To analyze the transcriptomes, 2 ml of cultures was collected and centrifuged for 15 s at 13,000 rpm. The supernatants were removed, and the tubes were immediately frozen in liquid nitrogen and stored at −80°C before RNA extraction. Cells were disrupted by a mixer mill (MM400; Retsch GmbH, Haan, Germany) run at a frequency of 30 s^{-1} for 60 s. RNA was isolated using the acid hot phenol-lithium chloride (LiCl) method as described Aberger et al. (19). Quantification of RNA was carried out using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE), and the quality of RNA was evaluated on an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) using an RNA 6000 nanochip kit, RNA ladder, and Agilent analysis software (Agilent Technologies).

One microgram of total RNA was used for the poly(A) tailing reaction (Ambion/Applied Biosystems). Poly(A)-labeled RNA was *in vitro* transcribed and labeled with cyanine 3 (Cy3; Invitrogen, Carlsbad, CA) and cyanine 5 (Cy5; Invitrogen) using an amino allyl MessageAmp II aRNA amplification kit (Ambion/Applied Biosystems). RNA was analyzed by microarrays (8 × 15K; Agilent, Santa Clara, CA) designed on the *L. gasicomitatum* LMG18811^T genome (4) using the Oligoarray (version 2.1) web tool (20). Two hundred micrograms of each differentially labeled amino allyl RNA (aaRNA) was fragmented at 60°C for 30 min using a Gene Expression hybridization kit (Agilent). Hybridization was performed at 65°C with rotation at 20 rpm for 16 h. Prior to scanning, the microarray was washed with washing buffers, following the instructions of the manufacturer (Agilent), and dried immediately by brief centrifugation (20 s, 1,000 × *g*). The microarrays were scanned with an Axon 4200 AL scanner (Axon Instruments, Inverurie, United Kingdom) using 5-μm pixel resolution, and the images were analyzed with GenePix (version 6.0) software (Axon). Data normalization and statistical analysis were performed using the statistical software tool R with the package limma (21). Alteration in gene expression was considered significant if the *P* value was ≤0.01. Microarray data are MIAMI (minimum information about a microarray experiment) compliant.

Pork inoculation experiment. A fresh (slaughtered <24 h earlier) whole pork (*Longissimus dorsi*) loin surface was decontaminated under boiling water for 30 s and then sliced into approximately 15-g slices. *L. gasicomitatum* strains LMG18811^T (wild type) and LMG18811^T::pSIP1333A (*cydB* mutant) were inoculated individually onto the pork. Inocula were prepared from MRS broth cultures in the middle exponential growth phase (16 to 18 h at 25°C) and subsequently diluted in 0.1% peptone saline to reach the final cell density of approximately 10^5 CFU ml^{-1}. The cultures were spread evenly onto the surface of the pork pieces (0.5 ml on both sides). Three independent replicates and uninoculated samples were done.

All pork cuts were packaged individually (Multivac A 300/168; Sepp Haggenmuller KG, Wolfertschwenden, Germany) in a modified atmosphere containing 20% CO_2 and 80% O_2 or N_2 using a high-barrier film 90 μm thick with an oxygen transmission rate of 1 cm^3 m^{-2} 24 h^{-1} atm^{-1} at 23°C and 75% relative humidity (Finnvacum, Östersundom, Finland). Packages were stored at +6°C in the dark. Two packages were analyzed every second or third day during the 29-day storage time.

The gas compositions in the packages were measured using a gas sensor (Checkpoint; PBI Dansensor, Ringstedt, Denmark). The pH of the pork was measured after 1 min homogenization (Stomacher; Seward, Worthing, United Kingdom) with 0.1% peptone saline at a ratio of 1:10 by a pH meter (Inolab 720; WTW, Weilheim, Germany).

Colony counts of pork samples were performed by homogenizing 10 g of pork with 0.1% peptone saline using a stomacher, and 10-fold dilutions were plated on MRS agar with or without 3 μg g^{-1}erythromycin (Sigma-Aldrich Co., St. Louis, MO). Erythromycin was used for separating the *cydB* mutant. Homogenates of uninoculated controls were spread on MRS agar and plate count agar (PCA; Oxoid). PCA plates were incubated aerobically at 25°C for 5 days. To verify the growth of the inoculants, up to 10 colonies were randomly picked from the MRS plates sampled on the day of spoilage and identified by numerical analyses of the HindIII ribotype (22).

Sensory analyses were performed by a trained panel of six individuals. For the analyses, the pork samples were equilibrated to room temperature. Fresh pork from the same meat batch used for the inoculated samples was stored in the freezer and used as a reference. The panelists evaluated the odor and the appearance of the samples using a five-point scale (1, severe defect, spoiled; 2, clear defect, spoiled; 3, mild defect, satisfactory; 4, good; 5, excellent) the observed deficiencies were described. The sample was considered spoiled when the median of the odor grades given was 2 or less.

Estimation of *L. gasicomitatum* growth parameters in MRS and in pork. The DMfit program (Institute of Food Research, Norwich, United Kingdom) was used for calculating the maximum growth rates (μ_{max}s) and maximum bacterial levels (N_{max}s) in MRS and pork by matching the colony counts and OD_{600} values with the growth model (23).

TABLE 2 Maximum growth rates and maximum bacterial levels of the 12 *Leuconostoc* strains[a]

L. gasicomitatum strain	Aerobic atmosphere				Anaerobic atmosphere			
	MRS		MRS + 2 μg heme ml^{-1}		MRS		MRS + 2 μg heme ml^{-1}	
	μ_{max}	N_{max}	μ_{max}	N_{max}	μ_{max}	N_{max}	μ_{max}	N_{max}
LMG18811^T	0.32 ± 0.01	0.93 ± 0.03	0.48 ± 0.02	1.27 ± 0.04	0.48 ± 0.02	1.21 ± 0.02	0.43 ± 0.01	1.26 ± 0.02
LMG18811^T::pSIP1333A	0.29 ± 0.01	0.87 ± 0.02	0.23 ± 0.02	0.82 ± 0.02	0.49 ± 0.02	1.18 ± 0.03	0.45 ± 0.01	1.23 ± 0.04
RSNS1b	0.17 ± 0.02	0.70 ± 0.01	0.31 ± 0.01	1.02 ± 0.03	0.34 ± 0.06	1.05 ± 0.02	0.37 ± 0.04	1.12 ± 0.03
N20-1	0.31 ± 0.01	0.95 ± 0.02	0.42 ± 0.05	1.21 ± 0.05	0.36 ± 0.10	0.96 ± 0.19	0.39 ± 0.06	0.98 ± 0.22
HHSL3c	0.23 ± 0.01	0.70 ± 0.01	0.34 ± 0.02	0.88 ± 0.02	0.46 ± 0.11	1.06 ± 0.20	0.44 ± 0.04	1.13 ± 0.16
ITTJA1a	0.32 ± 0.02	0.89 ± 0.04	0.39 ± 0.02	1.05 ± 0.02	0.40 ± 0.11	1.10 ± 0.11	0.37 ± 0.11	1.19 ± 0.05
POK13-6	0.17 ± 0.01	0.71 ± 0.01	0.22 ± 0.02	0.80 ± 0.01	0.33 ± 0.06	1.14 ± 0.03	0.26 ± 0.05	1.14 ± 0.05
NAPA6-6	0.20 ± 0.01	0.75 ± 0.01	0.29 ± 0.03	1.22 ± 0.02	0.37 ± 0.11	1.04 ± 0.04	0.40 ± 0.10	1.12 ± 0.03
NAGRM3a-2	0.36 ± 0.01	0.89 ± 0.02	0.43 ± 0.01	1.16 ± 0.02	0.43 ± 0.06	1.09 ± 0.03	0.39 ± 0.06	1.14 ± 0.04
NALP2-7	0.23 ± 0.02	0.89 ± 0.03	0.45 ± 0.01	1.22 ± 0.01	0.38 ± 0.02	1.12 ± 0.06	0.39 ± 0.02	1.24 ± 0.02
SISU-4-3	0.21 ± 0.01	0.83 ± 0.02	0.32 ± 0.01	1.06 ± 0.02	0.41 ± 0.01	1.08 ± 0.06	0.40 ± 0.01	1.06 ± 0.01
RSNL2b	0.18 ± 0.02	0.76 ± 0.01	0.39 ± 0.02	1.21 ± 0.01	0.39 ± 0.02	1.05 ± 0.02	0.37 ± 0.04	1.12 ± 0.03

[a] *Leuconostoc* strains were incubated in broth cultures in aerobic (ambient) and anaerobic (5 to 10% H_2, 10% CO_2, 80 to 85% N_2) atmospheres at 6°C in Bioscreen C. The values represent the means ± standard deviations of the 6 biologically replicate cultures.

VOCs of medium and pork. *L. gasicomitatum* broth cultures in MRS were collected for analysis of volatile organic compounds (VOCs) at the same time that the samples were collected for transcriptome analyses. From the inoculated pork, samples were collected at the time of inoculation, after 8 days of storage, and at the time of spoilage. Two milliliters of MRS and 3 g of meat were collected for analyses. The meat was cut into small (approximately 0.2-g) pieces. Samples were collected in 10-ml-headspace vials, an internal standard (67 ng g^{-1} 3,3-dimethyl-2-butanol; Sigma-Aldrich Co.) was added, and the vials were sealed and stored at −20°C before analysis. Two or three biological replicates were analyzed. VOCs were adsorbed from sample vials on a 75-μm carboxen/polydimethylsiloxane (CAR-PDMS) fiber. Prior to collection, the fiber was preconditioned at 300°C for 30 min in the gas chromatograph (GC) injection port. The sample vials were first equilibrated at 60°C in a water bath for 30 min, and after that, the fiber was exposed to the headspace above the sample at 60°C for 30 min. Samples were analyzed using an Agilent 6890N GC and an Agilent 5975B mass-selective (MS) detector. The capillary column was a DB-5ms apparatus (30 m by 0.25 mm [inner diameter]; film thickness, 0.25 μm; J&W). After extraction, the solid-phase microextraction (SPME) device was removed from the sample bottle. Manual injection was performed at 270°C, and the compounds were desorbed from the fiber for 5 min. To improve the peak shape of compounds that eluted early, the cold-trapping technique was used. During the desorption, the coil of the column taken from the injector was immersed in a Dewar vessel filled with liquid nitrogen. The split valve was opened after 5 min, and the compounds were desorbed from the cold trap by removing the coil of the column from the Dewar vessel and putting it back into the oven. The fiber was kept in the injection port during the whole GC run. The column temperature was programmed to increase from 40°C (isothermal time, 4 min) to 160°C at 5°C/min and to 280°C at 10°C/min, and the fiber was kept at the final temperature for 5 min. The injector temperature was 290°C, and the carrier gas was helium with a flow rate of 35 cm/s at 40°C. The transfer line between the GC and MS detector temperature was kept at 290°C. The ionization mode was electron impact (electron energy, 70 eV). The mass range used was from 29 to 250 atomic mass units (amu) with a scan rate of 3.16 scans per second. After each run, the fiber was cleaned up to avoid carryover, and the cleanness was periodically tested with an empty vial. All analyses were run using the same fiber unit. For the identification of VOCs, the National Institute of Standards and Technology (NIST) library was used. The detected VOCs were expressed in arbitrary units (AU) with 3,3-dimethyl-2-butanol as the internal standard. Total ion chromatograms (TICs) were used for the quantitation of volatile compounds.

Microarray data accession number. The microarray data have been deposited in the Gene Expression Omnibus (GEO) database under accession no. GSE41281.

RESULTS

Determination of respiration potential of *L. gasicomitatum* in broth culture. Under aerobic conditions, the *L. gasicomitatum* strains (Table 1) had 23% to 46% higher growth rates and 12% to 39% higher final OD_{600} values in heme-containing MRS broth than in broth without heme (Table 2). The addition of heme had no effect on anaerobically growing cells. The results show that the combination of oxygen and heme enhanced the growth of all the *L. gasicomitatum* strains tested ($P < 0.001$) and not only the growth of the type strain. The response of the *cydB* mutated strain (LMG18811^T::pSIP1333A) to heme was also studied. Under aerobic conditions, heme had no effect on the *cydB* mutated strain, showing that the heme-based respiration was successfully knocked out. Under anaerobic conditions, the *cydB* mutant and wild type (LMG18811^T) showed similar growth (Table 2), indicating that the genetic modifications of the mutant did not affect the genes/pathways functional during anaerobic fermentation.

Growth of *L. gasicomitatum* in pork. The growth of *L. gasicomitatum* LMG18811^T (wild type) was compared to the growth of LMG18811^T:pSIP1333A (*cydB* mutant) in pork stored under MAP conditions with or without oxygen at +6°C for 29 days. The growth curves showed that the *cydB* mutant had lower μ_{max} and N_{max} values (μ_{max}, 0.37 ± 0.21; N_{max}, 7.7 ± 0.21) than the wild type (μ_{max}, 0.65 ± 0.24; N_{max}, 9.01 ± 0.24) on pork stored under the high-oxygen modified atmosphere (Fig. 1). Under anaerobic conditions, the μ_{max} and N_{max} values of the *cydB* mutant and the wild type did not show significant differences (μ_{max}s, 0.58 ± 0.24 and 0.63 ± 0.17, respectively; N_{max}s, 8.3 ± 0.24 and 8.49 ± 0.17, respectively). Under aerobic conditions, the *cydB* mutant and the wild type reached N_{max} after 15 days and 11 days, respectively. After reaching N_{max}, the mutant's viable counts started to decrease under aerobic conditions. Under anaerobic conditions, both the mutant and the wild type reached N_{max} after 11 days. Overall, the results show that oxygen increased the growth rate and biomass of

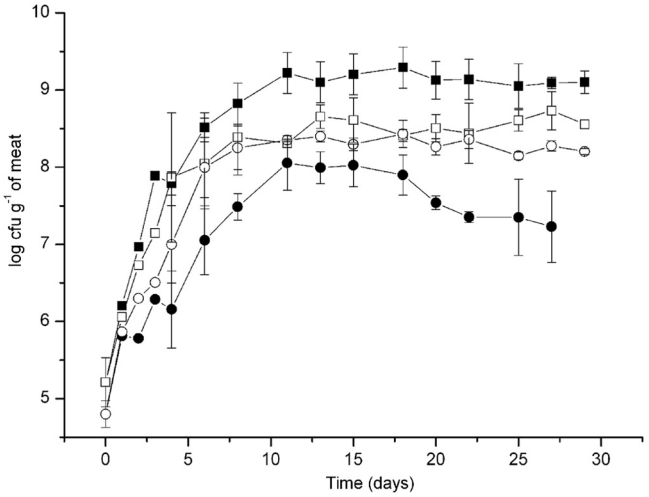

FIG 1 Colony counts of *L. gasicomitatum* LMG18811^T wild type (■, high oxygen; □, anaerobic) and *cydB* mutant LMG18811^T::pSIP1333A (●, high oxygen; ○, anaerobic) inoculated individually onto pork and stored at +6°C. The bars indicate the standard deviations of three replicates.

wild-type *L. gasicomitatum* LMG18811^T but decreased the growth rate, biomass, and survival of the *cydB* mutant with a deficient respiration chain.

At the beginning of the experiments, the pH was 5.5 ± 0.2, and on the final day it was slightly lower (5.2 ± 0.1). The respiration capacity of the strain or the packaging atmosphere did not affect pH development. On day 0, the initial background microbiota was <10 CFU g^{-1} of pork, and on the day that the maximum viable counts in the inoculated pork samples were reached, the viable counts (measured with MRS and PCA) in the uninoculated pork samples were approximately 10^3 CFU g^{-1}, which was ≤0.01% of the counts in inoculated samples. These results indicate that the concentration of background microbiota in inoculated meat was likely to have been too low to affect the results, since spoilage is usually detected when the counts are $>10^7$ CFU g^{-1}. The random isolates collected from the inoculated pork at the time of spoilage were identified to be the ribotype of the inoculated strain, which thus dominated in the packages (see Fig. S1 in the supplemental material).

Sensory analysis. According to the sensory panel, the odor of pork inoculated with the wild type and stored under high-oxygen MAP was considered spoiled after 13 days in all three replicates. Pork inoculated with the *cydB* mutant was evaluated to be spoiled after 13, 15, or 17 days, depending on the experiment. The spoiled odor was described as buttery and sour in all cases. The appearance of high-oxygen MAP meat was considered unacceptable (gray or green) after 13 days of storage, irrespective of the strain inoculated. The odor and appearance of anaerobically packaged pork were evaluated as spoiled after 24 to 29 days of storage. The inoculant (wild type or *cydB*) did not affect the sensory shelf life as much as the atmosphere. Meat samples stored under the anaerobic atmosphere spoiled about 10 days later than the corresponding meat samples stored under high-oxygen MAP.

VOCs measured by SPME-GC-MS detector. The maximum standard deviation between biological replicates was 40% in culture medium and 60% in pork. The bacterial metabolite concentrations increase in a logarithmic scale, whereas the chemical reactions (for example, lipid oxidation) occur linearly.

The addition of heme to the culture medium (MRS) with wild type increased 6- to 18-fold the concentrations of 2,3-butanedione (diacetyl) and 3-hydroxy-2-butanone (acetoin) under aerobic conditions (Table 3). Heme did not affect the concentrations of these compounds in broth cultures of the *cydB* mutant, showing that the accumulation of these compounds was enhanced by respiration. The concentration of ethanol, the end product of fermentative metabolism, was 10 times higher when wild type was grown without heme. The addition of heme did not affect the ethanol production of the *cydB* mutants, indicating that growth was based only on fermentative metabolism, despite the presence of heme. Other compounds listed in Table 3 did not seem to be connected to respiration.

TABLE 3 VOCs detected by SPME-GC-MS detector from broth after 16 h of aerobic growth of *L. gasicomitatum* LMG18811^T (wild type) and LMG18811^T::pSIP1333A (*cydB* mutant) at 25°C

Compound	Amt of VOC[a]			
	Wild type		*cydB* mutant	
	MRS	MRS + 2 μg ml^{-1} heme	MRS	MRS + 2 μg ml^{-1} heme
Alcohols				
Ethanol	93.0	9.7	112.0	92.7
1-Butanol	ND	ND	ND	13.9
2,3-Butanediol	ND	ND	ND	ND
3-Methyl-1-butanol	129.2	118.9	55.1	63.8
Aldehydes				
3-Methylbutanal	104.8	102.3	57.0	50.3
Butanal	ND	9.9	ND	ND
Benzaldehyde	5.1	5.9	5.7	4.7
Benzene acetaldehyde	0.7	0.4	1.0	0.8
Ketones				
Acetone	43.4	40.5	49.6	34.4
2,3-Butanedione (diacetyl)	8.8	55.3	8.0	7.7
3-Hydroxy-2-butanone (acetoin)	ND	18.4	ND	ND
2-Butanone	11.9	10.1	10.0	12.4
2-Heptanone	4.8	11.6	1.3	0.7
Organic acids, acetic acid	275.1	333.7	215.9	238.7
Aromatic hydrocarbons				
2,5-Dimethylpyrazine	33.5	77.3	32.9	26.4
Pyrazine	5.6	6.9	5.9	4.8
Trimethylpyrazine	2.9	3.2	3.2	2.9
3-Ethyl-2.5-dimethylpyrazine	0.6	0.7	0.8	0.6
Xylene	13.7	11.8	6.4	26.4
Vinylpyrazine	2.4	1.1	0.9	1.0
Esters, butyl acetate	ND	ND	ND	ND
Sulfur compounds				
Dimethyl sulfoxide	75.3	59.1	78.4	60.2
Dimethyl sulfide	13.2	27.3	10.6	2.0

[a] The values are reported as arbitrary units. TICs were used for quantitation of VOCs. The maximum dispersion between biological replicates (two or three) was 40%. ND, not detected.

TABLE 4 VOCs detected by SPME-GC-MS detector from meat inoculated with *L. gasicomitatum* LMG18811^T (wild type) and LMG18811^T::pSIP1333A (*cydB* mutant) at 6°C

Compound	Amt of VOC[a]											
	Wild type						*cydB* mutant					
	High oxygen			Anaerobic			High oxygen			Anaerobic		
	0 days	8 days	13 days	0 days	8 days	24–29 days	0 days	8 days	13–17 days	0 days	8 days	24–26 days
Alcohols												
Ethanol	75.0	26.9	16.9	75.0	93.0	326.0	102.0	59.9	158	102.0	92.1	475.4
1-Butanol	0.6	0.6	0.6	0.6	0.6	0.6	ND	3.2	ND	ND	ND	ND
2,3-Butanediol (diacetyl)	ND	138.9	330.3	ND	10.1	ND	ND	ND	ND	ND	ND	ND
1-Pentanol	14.3	26.8	27.0	14.3	17.4	10.7	13.5	12.1	19.8	13.5	7.2	9.7
2-Butoxyethanol	ND	12.9	2.4	ND	0.8	ND	ND	ND	15.1	ND	ND	4.3
3-Methyl-3-buten-1-ol	ND	ND	ND	ND	ND	ND	8.3	6.4	3.6	8.3	5.6	4.5
Isopropanol	6.7	15.3	2.9	6.7	8.7	9.8	7.7	20.8	12.3	7.7	17.3	21.3
2-Methyl-2-propanol	43.8	15.9	3.7	43.8	26.8	14.4	54.0	21.1	10.0	54.0	24.7	13.6
1-Hexanol	<1.0	20.2	24.2	<1.0	11.4	19.1	<1.0	<1.0	37.8	<1.0	<1.0	10.7
Aldehydes												
3-Methylbutanal	3.0	5.5	7.7	3.0	7.9	9.2	2.8	5.2	11.9	2.8	3.8	6.3
Hexanal	97.9	374.6	707.3	97.9	164.6	192.1	94.3	105.7	393.3	94.3	102.5	102.7
Octanal	2.0	5.8	6.2	2.0	2.6	4.6	2.1	2.6	8.0	2.1	3.0	2.6
Nonanal	4.7	12.7	1.0	4.7	3.1	6.6	5.6	5.4	8.6	5.6	6.3	4.7
Pentanal	14.3	40.9	76.8	14.3	16.1	19.1	11.9	14.1	50.0	13.2	10.6	10.7
2-Methyl cyclohexanal-*cis*	ND	11.3	11.3	ND	8.3	6.4	ND	5.7	11.8	ND	5.2	8.7
2-Methyl cyclohexanal-*trans*	ND	6.9	7.0	ND	5.7	4.2	ND	3.7	6.9	ND	3.3	4.7
Heptanal	2.9	11.6	23.2	2.9	5.6	9.3	3.0	4.9	16.5	3.0	4.7	8.1
2-Methylpropanal	7.0	8.2	4.4	7.0	2.3	6.0	6.5	7.1	8.6	6.5	ND	5.9
Benzaldehyde	11.9	13.3	23.6	11.9	5.5	11.5	9.2	8.4	17.6	9.2	8.8	17.8
Ketones												
Acetone	21.1	15.0	10.7	21.1	12.6	11.5	12.3	25.6	16.4	12.3	24.5	31.9
2,3-Butanedione	2.1	380.3	633.8	2.1	7.5	58.4	3.2	1.2	2.6	3.2	1.0	4.1
3-Hydroxy-2-butanone (acetoin)	9.2	1,359.0	2,683.1	9.2	19.6	89.6	ND	ND	ND	ND	ND	3.9
2-Butanone	17.3	3.0	28.7	17.3	10.5	32.3	ND	23.1	15.0	ND	19.6	21.0
2-Pentanone	4.8	23.7	33.1	4.8	6.7	5.8	4.4	12.3	5.1	4.4	7.4	3.0
1,3-Pentadiene	5.8	ND	ND	5.8	1.5	ND	0.6	2.4	3.2	0.6	2.8	3.0
3-Hepanone	2.5	9.7	9.7	2.5	7.2	5.9	2.4	6.7	10.5	2.4	6.6	4.7
Organic acids												
Acetic acid	9.9	261.0	610.8	9.9	44.7	82.7	9.1	23.4	99.2	9.1	5.7	160.3
Butanoic acid[b]	9.8	42.8	46.9	9.8	13.1	25.4	7.0	10.8	18.7	7.0	11.5	27.2
Hexanoic acid[b]	4.8	11.9	24.1	4.8	6.3	7.9	3.8	4.1	4.1	3.8	4.1	4.1
Aromatic hydrocarbons, toluene	1.9	2.7	2.9	1.9	2.2	2.3	1.8	5.3	3.8	1.8	4.0	5.8

[a] The values are reported as arbitrary units. TICs were used for quantitation of volatiles. The maximum dispersion between biological replicates (two or three) was 60%. ND, not detected.

[b] The chemical coeluted totally or partly with an interfering chemical. The quantities were estimated by using a characteristic ion which was not present in the interfering background chemical for the calculations. The calculation was based on the relative percentage of this ion of all ions in the spectrum obtained from the TIC of a clean peak of this chemical. By using this relative value the corresponding TIC value was calculated. This calculated TIC value was used to estimate the amount of coeluting chemicals by comparing the calculated and measured TIC values.

Table 4 shows the VOCs detected from pork. The colony counts under a high-oxygen atmosphere at the time of spoilage were 10^9 CFU g^{-1} (wild type) or 10^8 CFU g^{-1} (*cydB* mutant). In anaerobically stored pork, the colony counts were 10^8 CFU g^{-1} at the time of spoilage. After 13 days of storage under high-oxygen atmospheres, the levels of 2,3-butaedione (diacetyl), 3-hydroxy-2-butanone (acetoin), and 2,3-butanediol were 16 to 2,600 times higher in the samples inoculated with the wild type than in the samples inoculated with the *cydB* mutant. Under anaerobic conditions, accumulation of these compounds was not detected after 24 days of storage. Acetic acid production was also 6 times higher in wild-type cultures under high-oxygen atmospheres than under anaerobic conditions or in *cydB* mutant-inoculated meat. During aerobic storage, the levels of hexanal, heptanal, and pentanal linearly increased about 6-fold, indicating lipid oxidation. 1-Hexanol and butanoic acid levels increased under both storage conditions. Variations in the concentrations of other compounds listed in Table 4 did not indicate any clear patterns. The results

TABLE 5 Main *L. gasicomitatum* genes with known function whose expression levels responded to respiration and diacetyl/acetoin genes with no response to respiration[a]

Function and gene	ORF description	Log FC ratio (*P* value)	
		Respiring[b] wild type[c]/ *cydB* mutant[d]	Respiring wild type[b]/ nonrespiring[e] wild type[c]
Transport and metabolism			
LEGAS_0186, *nhaC*	Na/H antiporter	2.0 (0.01)	1.7 (0.006)
LEGAS_0271, *araE*	Arabinose-proton symporter	−1.9 (0.003)	NS
LEGAS_0324, *treR*	Trehalose operon repressor	−2.0 (0.007)	NS
LEGAS_0325, *treB*	PTS system, trehalose-specific IIBC component	NS	−1.7 (0.01)
LEGAS_0527, *nrdD*	Anaerobic ribonucleoside triphosphate reductase	−1.3 (0.007)	−1.6 (0.002)
LEGAS_0528, *nrdG*	Anaerobic ribonucleoside triphosphate reductase activating protein	−1.8 (0.003)	−2.2 (0.0007)
LEGAS_0617	PTS lactose/cellobiose IIB component	−2.1 (0.0009)	NS
LEGAS_0734, *cutC*	Copper homeostasis protein	2.5 (0.0009)	NS
LEGAS_0941, *mleS*	Malolactic enzyme	NS	−3.2 (4.8e−6)
LEGAS_0942, *mleP*	Na/malate symporter	−1.5 (0.01)	−2.6 (3.5e−5)
LEGAS_1101, *amt*	Ammonium transporter	1.8 (0.01)	2.2 (0.004)
LEGAS_1274, *mraY*	Phospho-*N*-acetylmuramoyl-pentapeptide transferase	1.9 (0.002)	NS
LEGAS_1458, *glnQ2*	Glutamine transport ATP-binding protein	2.2 (0.002)	NS
LEGAS_1624, *nagB*	Glucosamine-6-phosphate deaminase	−1.4 (0.001)	−1.5 (0.004)
LEGAS_1892	Permease of the major facilitator superfamily	NS	1.7 (0.009)
Repair, LEGAS_1002, *uvrX1*	UV-damage repair protein	−1.8 (0.01)	−1.9 (0.0008)
Phage-related function			
LEGAS_1589	Phage protein, putative	NS	−1.4 (0.009)
LEGAS_1590	Phage portal protein, SPP1 family	NS	−1.3 (0.01)
LEGAS_1600	Prophage Lp1 protein 24	−3.7 (4.1e−6)	−2.3 (0.0009)
LEGAS_1605	Sak2	−2.7 (0.007)	−2.5 (0.001)
LEGAS_1609	Prophage protein	−2.1 (0.002)	−1.8 (0.004)
LEGAS_1614	Phage antirepressor	−2.4 (0.002)	−2.4 (0.002)
Diacetyl/acetoin pathway			
LEGAS_0526, *alsS*	Acetolactate synthase, catabolic	NS	NS
LEGAS_0299, *butA1*	Acetoin(diacetyl) reductase	NS	NS
LEGAS_1299, *butA2*	Acetoin(diacetyl) reductase	NS	NS
LEGAS_1346, *alsD*	Alpha-acetolactate decarboxylase	NS	NS
LEGAS_1018, *bdhA*	(*R*,*R*)-Butanediol dehydrogenase	NS	NS

[a] The values were calculated by using the statistical software tool R with the package limma, and the data were transferred to the Microsoft Excel program. Five probes were used, and the median of the log fold change (log FC) and *P* values are shown. The negative values indicate downregulation. ORF, open reading frame; NS, not significant (*P* value of >0.01 and also a log FC ratio of <1.3); PTS, phosphotransferase.
[b] MRS containing heme at 2 μg ml^{-1}.
[c] The wild type was $LMG18811^T$.
[d] The *cydB* mutant was $LMG18811^T$::pSIP1333A.
[e] MRS without heme.

show that both the storage atmosphere and the respiration capacity of the *L. gasicomitatum* strain inoculated influence the volatile profiles of pork meat.

Transcriptome analysis attributable to respiration. Respiration showed differential expression for 22 genes with known functions (Table 5) and for 17 genes without a known function (see Table S1 in the supplemental material). The major changes introduced by respiration in transcriptomes were the decrease in the expression levels of the genes with phage-related functions. No differences in expression levels for diacetyl/acetoin pathway genes were noticed (Table 5). Compared to the respiring wild type, the gene expression levels in the nonrespiring wild type (cultivated without heme) and in the *cydB* mutant (cultivated with or without heme) showed quite similar gene expression patterns (Table 5). This showed that respiration affected gene expression in a quite similar way, irrespective of whether the nonrespirative state was achieved using mutation or deprivation of heme from the culture medium. No significant ($P < 0.01$) gene expression differences were noticed when the nonrespiring wild type was compared to the *cydB* mutant or when the mutant cultivated with heme was compared to the mutant cultivated without heme (Table 6). These results showed that the mutation in the *cydB* gene did not significantly affect the expression levels of other genes.

DISCUSSION

Many LAB are genetically equipped for aerobic respirative metabolism when exogenous heme is available (13). The aerobic respiration ability of *L. gasicomitatum* type strain LMG18811 has been detected by Johansson et al. (4). In the current study, we showed that this capability is not limited only to the *L. gasicomitatum* type strain. The respiration has been detected to improve biomass and

TABLE 6 Number of genes differing significantly in expression levels between wild type (LMG18811^T) and *cydB* mutant (LMG18811^T::pSIP1333A) strains of *L. gasicomitatum* cultivated with or without heme in MRS broth aerobically

Strain	No. of genes			
	Wild type with heme	Wild type without heme	*cydB* mutant with heme	*cydB* mutant without heme
Wild type with heme	0			
Wild type without heme	16	0		
cydB mutant with heme	17	0	0	
cydB mutant without heme	17	0	0	0

afford greater long-term survival of lactococci (12, 24). The metabolism of lactococci seems to be biphasic under respiration conditions: growth first takes place mainly via fermentation and then takes place via respiration (12). Besides the higher biomass, we also showed that respiration increased the growth rate of *L. gasicomitatum*, indicating that respiration has an early beneficial effect on the growth. To our knowledge, the positive effect of heme on the growth rate in LAB has not been published earlier.

In this study, growth based on respiration in meat with endogenous heme was found to be remarkable in terms of population development and subsequent development of sensory characteristics. We created and compared three meat spoilage models with *L. gasicomitatum*: (i) aerobic with fermentation and respiration, (ii) aerobic with only fermentative metabolism, and (iii) anaerobic with only fermentative metabolism. Under high-oxygen MAP, the growth competence (μ_{max} and N_{max}) of *L. gasicomitatum* LMG18811^T based on respiration and fermentation (model i) improved significantly in comparison to the metabolism based only on fermentation (model ii). The *L. gasicomitatum* colony counts in meat (Fig. 1) indicated that under aerobic conditions, respiration may accord better long-term survival for *L. gasicomitatum*. This, however, appeared after the end of the sensory shelf life of the meat and was therefore insignificant, considering commercial manufacture. The respiration effect on the long-term survival of *L. lactis* (12) was more pronounced than that observed for *L. gasicomitatum* in the present study.

Respiration causes several changes in LAB metabolism, including decreased production of lactic acid and increased production of acetate, acetoin, and diacetyl (12, 25). Diacetyl is a major flavor metabolite produced by LAB and imparts a buttery aroma and flavor to many fermented foods (26). However, in meat, this metabolite, together with acetoin, has been considered to have a major sensory spoilage attribute (27, 28). In our study, the production of acetoin and diacetyl by *L. gasicomitatum* was minimal in meat packaged under anaerobic conditions (model iii) and under high-oxygen conditions when growth was based only on fermentative metabolism (model ii). Respiration enormously increased the accumulation of acetoin and diacetyl under a high-oxygen modified atmosphere (model i).

However, spoilage cannot be predicted on the basis of growth or chemical analyses of compounds without sensory analysis. According to the sensory panel odor evaluation, the *cydB* mutant strain, dependent on fermentation, spoiled meat only 2 days later than the wild-type strain, even though the mutant N_{max} values were only 1% of those measured for the respiring wild type under aerobic conditions. The sensory evaluation panel did not respond to the observed differences in diacetyl/acetoin levels. The anaerobically stored meat was evaluated to be spoiled about 10 days later than the meat under high-oxygen MAP, despite the diacetyl/acetoin levels, showing that the atmosphere influenced the sensory shelf life of meat much more than the respiration capacity of the inoculated *L. gasicomitatum* strain. Our observations emphasize the fact that the meat spoilage odor comprises a mixture of VOCs generated by bacterial metabolism and lipid oxidation. On the other hand, under anaerobic conditions, the meat quality remained acceptable for 13 to 18 days after *L. gasicomitatum* LMG18811^T had reached the maximum viable counts. Under aerobic conditions, the meat was evaluated to be spoiled not more than a couple of days after the highest viable counts were reached.

The VOC levels were generally higher in high-oxygen MAP than in anaerobically stored pork. Ercolini et al. (29) also monitored mainly higher levels of VOCs from meat packed under high-oxygen MAP than under vacuum, except the levels of butanoic acid were clearly higher under vacuum. Butanoic acid can derive from the microbial consumption of free amino acids (30). This compound has been associated with the growth of LAB (29) and meat spoilage (31). In our study *L. gasicomitatum* produces similar levels of butanoic acid under both tested atmospheres (high oxygen, anaerobic) in pork. Another LAB, *Carnobacterium maltaromaticum*, has also been shown to produce nearly similar levels of butanoic acid both under vacuum and in air (32). Unlike *L. gasicomitatum* in our study, *C. maltaromaticum* has also been shown to produce acetoin without oxygen (32).

Only a few changes in gene expression are connected to respiration (13, 33, 34). Our transcriptome analyses showed that gene expression patterns were quite similar, irrespective of whether respiration was turned off by mutating the *cydB* gene or excluding heme from the medium. We observed respiration-induced changes in transporter activities similar to those observed by Pedersen et al. (34): upregulation of ammonium and downregulation of malate. In *L. lactis*, the *fhuDBAR* operon may involve heme uptake (24) and *ygfCBA* may be used for heme efflux (34). *L. gasicomitatum* LMG18811^T does not have those operons (4), and we did not detect any noteworthy candidate genes for heme uptake or efflux. Because of missing candidate genes, we did not confirm the results by reverse transcription-quantitative PCR. Despite the massive, respiration-induced increase in the production of diacetyl/acetoin, we did not detect any changes in the levels of expression of genes connected to the diacetyl/acetoin pathway. A similar observation made by Pedersen et al. (34) suggests that the fluxes away from pyruvate are probably controlled at the metabolic level rather than by gene expression.

Many of the *L. gasicomitatum* spoilage cases have been reported from high-oxygen packaged meats (2). Our results show that respiration is one key factor explaining why *L. gasicomitatum* is so well adapted in high-oxygen packed meat. Without respiration, oxygen has a negative effect on the growth of *L. gasicomitatum*, but with functional respiration, oxygen enhanced growth. The production of diacetyl and acetoin had a strong connection to the respiration of *L. gasicomitatum* in meat. These findings open new perspectives and increase our understanding of the *L. gasicomitatum* lifestyle in meat.

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