1 **Longitudinal metatranscriptomic analysis of a meat spoilage microbiome detects abundant**
2 **continued fermentation and environmental stress responses during shelf life and beyond.**

3

4

5 Running title: Longitudinal analysis of a meat spoilage microbiome

6

7 Jenni Hultman^{1,2}, Per Johansson¹, Johanna Björkroth^{1*}

8 ¹*Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University*
9 *of Helsinki, Agnes Sjöbergin katu 2, 00014 Helsinki University, Finland*

10 * Corresponding author

11 ² Current affiliation: Department of Microbiology, University of Helsinki, Finland

12 **Abstract**

13 Microbial food spoilage is a complex phenomenon associated with the succession of the specific
 14 spoilage organisms (SSO) over the course of time. We performed a longitudinal metatranscriptomic
 15 study on a modified atmosphere packaged (MAP) beef product to increase understanding of the
 16 longitudinal behavior of a spoilage microbiome during shelf life and onward. Based on the annotation
 17 of the mRNA reads, we recognized three stages related to the active microbiome that were descriptive
 18 for the sensory quality of the beef: acceptable product (AP), early spoilage (ES) and late spoilage
 19 (LS). Both the 16S RNA taxonomic assignments from the total RNA and functional annotations of
 20 the active genes showed that these stages were significantly different from each other. However, the
 21 functional gene annotations showed more pronounced difference than the taxonomy assignments.
 22 Psychrotrophic lactic acid bacteria (LAB) formed the core of the SSO according to the transcribed
 23 reads. *Leuconostoc* species were the most abundant active LAB throughout the study period, whereas
 24 the activity of Streptococcaceae (mainly *Lactococcus*) increased after the product was spoiled. In the
 25 beginning of the experiment, the community managed environmental stress by cold-shock responses
 26 which were followed by the expression of the genes involved in managing oxidative stress.
 27 Glycolysis, pentose phosphate pathway and pyruvate metabolism were active throughout the study at
 28 a relatively stable level. However, the proportional activity of the enzymes in these pathways changed
 29 over time. For example, acetate kinase activity was characteristic for the AP stage whereas formate
 30 C-acetyltransferase transcription was associated with spoilage.

31 **Importance**

32 It is generally known which organisms are the typical SSO in foods, whereas the actively transcribed
 33 genes and pathways during microbial succession are poorly understood. This knowledge is important
 34 since better approaches to food quality evaluation and shelf life determination are needed. Thus, we
 35 conducted this study to find longitudinal markers that are connected to quality deterioration. These

kind of RNA markers could be used to develop novel type of rapid quality analysis tools in the future. New tools are needed since even though SSO can be detected and their concentrations determined using the current microbiological methods, results from these analyses cannot predict how close timewise a spoilage community is from production of clear sensory defects. Main reason for this is that the species composition of a spoilage community does not change dramatically during late shelf life, whereas the ongoing metabolic activities lead to the development of notable sensory deterioration.

Introduction

There has been an increasing market trend for case-ready meat, i.e. meat that is processed, packaged, and labeled at a central meat processing facility and delivered to the retail store ready to be put directly into the meat case. From the microbial ecology perspective, these packages are man-made niches in which microbial succession leading to spoilage occurs under the selective pressures of cold and the modified atmosphere (MA) applied (1). In comparison to atmosphere, increased CO₂ levels (>20%) are used to limit the growth of aerobic food spoilage organisms, whereas high O₂ concentrations (70% to 80%) are needed to keep myoglobin oxygenated to ensure the red appealing color of beef.

Even though case-ready meat products have great market value, we know relatively little about which pathways are active in a developing spoilage community over the course of time during shelf life. The use of CO₂-containing atmospheres combined with refrigeration results in a dominance of psychrotrophic LAB, *Brochotrix thermosphacta* and, to some extent, *Enterobacteriaceae* in MAP meat (1, 2). During the last years, 16S rRNA gene amplicon-based approaches have increased the knowledge of community diversity in meat spoilage microbiomes (1, 3-9) or microbiota associated with food production environments (4, 7). However, no attention has yet been given to the activities related to the succession of a food spoilage community during shelf life.

We recently studied system level responses of three SSO during growth *in vitro* (10), i.e. *Leuconostoc gelidum* subsp. *gasicomitatum* (subsp. *gasicomitatum*), *Lactococcus piscium*, and *Paucilactobacillus oligofermentans*, by comparing their time course transcriptome profiles obtained during growth. The study revealed how these LAB employed different strategies to cope with the consequences of interspecies competition. The fastest-growing bacterium, subsp. *gasicomitatum*, enhanced its nutrient-scavenging and growth capabilities in the presence of other LAB through upregulation of carbohydrate catabolic pathways, pyruvate fermentation enzymes, and ribosomal proteins, whereas the slower-growing *L. piscium* and *P. oligofermentans* downregulated these functions (10). This current study prompted from the observations we made about the different behavior of the three LAB while growing as communities *in vitro* (10). We wanted to investigate if we can recognize similar phenomena in the behavior of the SSO growing on meat over the course of time.

To create a comprehensive view of the activities of the spoilage community over the course of time, we analyzed the metatranscriptomes of natural beef spoilage communities developing at +6°C. We wanted to show which metabolic pathways and defense responses are transcribed during microbial succession and spoilage stages in a commercial meat product. Our aim was to study if we can distinguish different stages related to the sensory deterioration using longitudinal metatranscriptomic analysis.

Results

We followed the development of a microbial community for 11 days using packages originating from the same production lot of a commercial MAP beef product manufactured by a large-scale operator. Production lot refers to a daily production and consist over 1 000 kg of meat. The experiment timespan covered the shelf-life and two days beyond. Altogether, a total of 65.8 Gb of sequence data (413.7 M reads) were obtained from the samples (Supplementary table S1). Good quality RNA suitable for

metatranscriptomic analyses was obtained from all samples from day 2 onwards. Alongside the total RNA and rRNA depleted RNA sequencing, the approaches included conventional microbiology, sensory and pH analyses associated with the meat quality.

Bacterial levels and sensory analysis results

On the first sampling day, bacterial levels were typical for a fresh product (LOG 4.6 and LOG 4.5 CFU/g LAB and total aerobic count, respectively) and at the end of the experiment the concentrations had increased to LOG 8.3 and 8.2 CFU/g for LAB and total aerobic bacteria, respectively (Fig. 1.). During the 11 days, the pH of the meat dropped slightly, from 5.9 to 5.5 (Supplementary table S2) and the microbial metabolism had also changed the packaging gas composition: O₂ content decreased from 74% to 57% and CO₂ increased from 20.5% to 37%, respectively. These changes are indicative for the activity of psychrotrophic heterofermentative spoilage LAB as anticipated.

For the bioinformatic analyses, the samples were divided into three groups based on the sensory analysis results (Fig. 1, Supplementary table S2) to enable detection of possible biomarkers associated with commercial meat quality during shelf life and beyond. The panelists started to deem the meat spoiled from day 5 on, with both odor and appearance receiving mean scores of 3/5. Samples assigned to the ES group (n=8 packages) received mean scores of 2.7/5 and 3.0/5 of odor and appearance, respectively. Samples receiving scores below 2 were assigned to the LS group and the mean odor and appearance scores were 1.75/5 and 1.65/5, respectively (Fig. 1, Supplementary table S2).

Active microbial community composition during shelf life and beyond

Taxonomic assignment of the 16S rRNA gene fragments picked from the total RNA fraction indicated that the active microbial communities were composed mainly of *Firmicutes* from the families of Leuconostocaceae and Streptococcaceae, and unclassified bacilli (Fig. 2). During the first 5 days of

the study (AP), Bacillales (unclassified bacilli and Bacillales) were active showing an abundance of 7.6% of the 16S rRNA gene transcripts but the abundance decreased beyond day 6 in ES. Leuconostocs were active throughout the experiment (Fig. 2) with abundance of 16.6-17.4%. In ES, the abundance of RNA transcripts of Streptococcaceae, mainly comprising sequences from the genus *Lactococcus*, increased to 5.2% of all detected 16S rRNA gene transcripts and this abundance remained high until the end of experiment, being 14.4% in LS. Based on the 16S rRNA results of the abundant genera and previous literature, we selected three SSO, i.e. subsp. *gasicomitatum* (LMG 18811⁺), *L. piscium* (MKFS47), *Dellaglioia algida* (DSM 15638⁺), for mapping of all reads against their genomes. The abundance of these psychrotrophic LAB was supported by the bowtie2 mapping since the majority of all RNA reads were assigned to these species (Table 1). The proportion of subsp. *gasicomitatum* was high from day 3 on (Table 1) whereas *L. piscium* reads increased on day 7 when the products had been deemed spoiled by the sensory panel. The reads mapping to *D. algida* showed highest abundance on day 4 (7.0 and 19.9%) but decreased during storage and thus this species was not very active during late shelf life and beyond (Table 1).

123

124 *Three stages in the viewpoints of taxonomy and functional gene annotations*

To explore the functional genes expressed over the course of time during shelf life, the sequenced mRNA fraction was analyzed. The results from read annotation against SEED subsystems database showed the community activity to change over time in the sample clusters related to stages AP, ES and LS. In NMDS plot of the Bray Curtis distance matrix, the samples clustered according to the stage. A permutation-based multivariate ANOVA (PERMANOVA) used for analyzing both the 16S rRNA gene taxonomic assignments and functional gene annotations revealed significant ($R^2=0.33$, $p<0.001$) effect of the stage for taxonomic grouping of the samples (AP, ES, LS; Fig 2). However, the differences were more pronounced for the functional gene annotations ($R^2=0.67$, $p<0.01$, Fig 2) than taxonomic assignments.

134 *Specific activities and changes in acceptable product*

135 Microbial community at the acceptable product (AP) stage expressed cold shock genes (Fig. 3),
 136 indicating temperature stress at +6°C storage conditions. Genes related to protein biosynthesis
 137 constituted from 16% to 22% of all reads during exponential growth while decreasing to 12% and
 138 9% in the later stages (Supplementary table S3). Active growth and succession of the community was
 139 thus strongly related to the stage while the product was still acceptable. According to SEED
 140 subsystems groups, cell division (1.1-1.25%, Supplementary table S3) was another function
 141 expressed significantly high during the exponential stage. These findings agree with the cultivation
 142 results indicating active community growth in the AP.

143
 144 During the AP, the abundance of gene transcripts clustering to "Carbohydrate metabolism" was one
 145 of the highest throughout the study and increased over time (Fig. 2). Detailed comparison of the
 146 carbohydrate metabolism in the three community growth stages showed that despite the rather similar
 147 expression level of the fermentation pathway, the relative abundance of genes transcribed in the
 148 pathways varied over time (Fig. 4, bubbles and pies related to glycolysis and pentose phosphate
 149 pathways). At the AP stage, the bacterial community required energy through fermentation via the
 150 pentose phosphate pathway (Fig. 4). Pyruvate metabolism was the second most abundant
 151 carbohydrate metabolism pathway during AP. However, unlike the spoilage stages, the majority of
 152 the transcripts were assigned as acetate kinase present in several different metabolic pathways. In the
 153 spoilage stages, the relative abundance of formate C-acetyltransferase, converting pyruvate to acetyl-
 154 CoA and formate, was significantly higher (Fig. 4.). Additionally, the third most abundant KO
 155 pathway was glycolysis, which had highly expressed genes throughout out the experiment (Fig. 4).

156
 157 Mapping of the transcripts to the three selected SSO genomes showed that cold shock and universal
 158 stress pathways were active during the AP stage (Supplementary table S4). In subsp. *gasicomitatum*,

the pentose phosphate pathway was expressed. In addition, amino acid synthesis was abundantly expressed. However, in *L. piscium*, genes related to lactate fermentation and glycolysis were expressed in relation to requirement of energy (Supplementary table S4).

Specific activities and changes in samples showing early spoilage

After day 6 during the ES stage, the cold shock genes were no longer expressed (Fig. 3), and the succession had apparently led towards the cold-tolerant species and strains. The 16S rRNA data shows an increase in the proportion of psychrotrophic LAB with a decrease in mesophilic species. On the functional side there was a change as well: the abundance of transcripts involved in reaction to oxidative stress increased together with genes involved in microbial respiration (Fig. 3).

The members of the ES microbial community expressed genes involved in respiration as the activities of different cytochrome oxidases and ubiquinone menaquinone-cytochrome c reductase complexes (Supplementary table S3) acting in electron donating and accepting reactions were found to be abundant. The pathways with higher abundance in expression compared to the those in AP included formation of sugar alcohols, fermentation and central carbohydrate metabolism (Supplementary table S3). In more detail, the microbial communities expressed genes for glycolysis and gluconeogenesis and pentose phosphate pathway (Fig. 4, Supplementary table S3) as well as different fermentative energy requirement reactions (Supplementary table S3). Although stress response as a whole increased only slightly over time, the genes involved in oxidative stress were found more actively expressed from day 6 onward (Fig. 3). Similarly to cold shock reactions, the abundance of gene transcripts involved in cell division and cell cycle, motility and chemotaxis, nucleoside and nucleotide utilization and protein metabolism decreased with time (Supplementary table S3). This indicated that the community was no longer growing actively.

183 In the ES stage, subsp. *gasicomitatum* was abundant (48% based on *Leuconostoc* 16S rRNA gene
184 abundance [Fig. 1] and 35% of the reads mapped to genome of subsp. *gasicomitatum* [Table 1]) and
185 based on mapping of the RNA transcripts to the genome the active pathways included pentose
186 phosphate pathway for energy requirement. The genes associated with stress and also the thioredoxin
187 reductase activity increased compared to AP. Thioredoxin reductase has been shown to be related to
188 survival in oxygen induced stress conditions (11) and this indicates that the species were actively
189 using mechanisms to tolerate the stress caused by the high oxygen MAP. In addition, heme-dependent
190 respiration of subsp. *gasicomitatum* (genes *cydA* and *cydB*) was found active in the AP and ES
191 (Supplementary table S3).

192

193 At the ES stage, the second most abundant LAB species, *L. piscium*, utilized noxE NADH oxidases
194 which can indicate a way to survive the redox stress in the high oxygen packaging during the active
195 growth phase. Additionally, the aldehyde-alcohol dehydrogenases (*adhE*) reported to possess
196 moonlight functions such as oxidative stress protection (12), were highly expressed (0.31+-0.21%).
197 Unlike the heterofermentative subsp. *gasicomitatum*, which uses a phosphoketolase pathway for
198 energy requirement, homofermentative *L. piscium* utilized glycolysis together with lactate and
199 pyruvate fermentation (Supplementary table S4). Additionally, in ES the cold shock genes were
200 highly active for *L. piscium* as well as the genes corresponding to sugar transporters. Moreover, in
201 *L. piscium* gene *yngB*, coding for the fibronectin-binding protein was expressed at the ES stage
202 (Supplementary table S4).

203

204 *Specific activities and changes associated with late spoilage*

205 In pentose phosphate metabolism, the fermentation of xylulose to pentose (genes *xfp*, *xpk*; xylulose-
206 5-phosphate/fructose-6-phosphate phosphoketolase) increased in the spoiled product, a phenomenon
207 which is associated with the production of pyruvate (Supplementary table S3). In addition, there was

208 increased activity of the pathway leading to the production of ethanol and acetate instead of common
209 spoilage volatiles diacetyl and 2,3-butanediol. Similarly, the activity of the pentose metabolism gene
210 *pflD* (formate C-acetyltransferase) increased in the LS stage. The gene catalyzes the reversible
211 conversion of pyruvate into formate and is thus involved in the supply of pyruvate to the pentose
212 phosphate pathway/metabolism.

213

214 The ES and LS stages differed in the utilization of di- and oligosaccharides and trehalose, which were
215 both significantly higher in after use-by date samples. Trehalose acts as a stress protectant and storage
216 carbohydrate and it is known that rapidly growing cells having lower trehalose levels than slow-
217 growing and stationary-phase cells (13), explaining the lower activity in exponential phase cells.
218 Malate metabolism increased significantly in the LS stage, especially the malate dehydrogenase,
219 citrate synthase and isocitrate dehydrogenase genes associated with formation of citrate. During LS
220 stage, starch metabolism was at its highest expression level (Fig. 4 bubbles) and also the relative
221 abundance of genes transcribed in relation to it varied greatly over time (Fig. 4 pies).

222

223 In addition to carbohydrate metabolism, amino acid metabolism (arginine, urea cycle and
224 polydeimines) increased with time as we found a seven-fold increase from ES to LS after the end-of
225 shelf life stage (Supplementary table S3). These genes include *arcA*, *arcB*, *arcC*, *arcT* and *arcD*,
226 corresponding to arginine deiminase, ornithine carbamoyltransferase, carbamate kinase, aspartate
227 aminotransferase and arginine/ornithine antiporter.

228

229 After the product was spoiled at the LS stage, the majority of the reads mapping to subsp.
230 *gasicomitatum* were involved in a pentose phosphate pathway producing pyruvate which, in the
231 presence of oxygen, has been reported to lead to production of diacetyl (9, 24), a spoilage metabolite,
232 through the chemical reaction of alpha acetolactate. As in ES, genes involved in general and oxygen-

233 induced stress were active. However, unlike in ES, proteolytic genes (*clpL*, ATP-dependent Clp
234 protease) became active and the abundance of reads mapping to sugar transporters increased. As was
235 seen at the microbial community level, the trehalose metabolism increased at this stage and the genes
236 were assigned to the main bacteria of this stage, subsp. *gasicomitatum*. For *L. piscium*, the metabolism
237 did not change between ES and LS stages.

238

239 Based on the Metaphlann2 annotation (14) Ascomycota, especially yeasts from genus
240 Debaryomycetaceae increased during shelf life (Fig. 5). The proportion of reads mapping to
241 Debaryomycetaceae was 1.49% ($\pm 2.37\%$) in the AP, 6.11% ($\pm 4.38\%$) in ES and increased to 25.62%
242 ($\pm 12.74\%$) in LS. Based on the MG-RAST annotations, the reads from LS mapping to
243 Debaryomycetaceae fungi had active energy metabolism/carbohydrate metabolism as genes involved
244 in glycolysis, glyoxylate cycle and acetate metabolism were among those expressed (Supplementary
245 table S5). Besides carbohydrate metabolism, fatty acid metabolism was active as the genes encoding
246 glycerol-3-phosphate dehydrogenase, stearyl-CoA desaturase and Acyl-CoA synthetase 2, to
247 mention a few, had RNA transcripts mapping to them (Supplementary table S5). The activity of these
248 yeasts was supported by finding RNA transcripts for several translation elongation factors as well as
249 ribosomal 60S and 40S subunit ribosomal proteins. In yeasts, the genes involved in respiration were
250 active too, and included different cytochrome C oxidases and oxoreductases as well as ferredoxin-
251 dependent glutamate synthase. In addition, transaldolase, a gene from the non-oxidative phase of the
252 pentose phosphate pathway, was active in yeasts during LS.

253 Discussion

254 Based on the clustering of active transcripts in NMDS and the observed sensory characteristics and
255 bacterial growth levels, the active microbiome and active genes in this MAP beef had three stages
256 that were descriptive for the quality of the product: acceptable product (AP), early spoilage (ES) and

late spoilage (LS). All these stages shared species and functions but there were several distinct metabolic and other responses associated with each of the three stages (Figs. 2 and 6). As typical for a packaged meat product (15), the microbial levels of the four stages did not directly correlate with the sensory quality of the product, whereas each of the four stages had specific activities based on the gene level activity analyses. Starting from the end of the AP stage, the same bacterial species remained active, something which highlights the reason why the active genes and pathways should be analyzed further instead of the active microbes to understand which metabolic pathways play a major role in food spoilage.

Even though leuconostocs, lactobacilli and lactococci have been associated with meat spoilage in several different studies using cultivation and DNA based approaches (16-19), little has been known about the most active functions during and after their succession. Based on the analysis of the active 16S rRNA genes in the total sequenced RNA fraction, leuconostocs were the most abundant bacteria throughout the study. However, the proportion of psychrotrophic *Lactococcus* increased after day 6. After shelf life (day 9), the LAB community was still active but the abundance of RNA transcripts of yeasts from the genus *Debaryomycetaceae* increased. Yeasts have not been considered to play a role in the spoilage of cold-stored meat before. Some cultivation-based studies have showed the fungi from *Saccharomycetaceae* phyla present in different meat environments, and also during the ripening of salami (20). The *Debaryomycetaceae* yeast increased drastically in the LS stage while the activity of the spoilage microbial community pertained to alcohol production from the pentose phosphate pathway. The yeasts were obviously active members of the fermentative community with up to 43.2% relative abundance in total microbial reads on the last day of the experiment (day 11) even though there was variation between different packages. Our results thus show that during long shelf-life, fungi and their spoilage functions increased in this MAP product.

282 Fermentation of carbohydrates was the most abundant metabolic activity detected in the samples over
 283 time as the prevalence of genes involved in carbohydrate metabolism was high throughout the study.
 284 The prevalence of actively expressed genes involved in carbohydrate metabolism shows that unlike
 285 in the spoilage of non-packaged meat caused by pseudomonads under atmosphere (21), the
 286 exhaustion of glucose is not a limiting factor for spoilage microbiota under MA containing 20%
 287 carbon dioxide and 80% oxygen. We observed activities of both homofermentative LAB, catabolizing
 288 glucose using the Embden–Meyerhof–Parnas (EMP) pathway, together with heterofermentative
 289 LAB, using the pentose phosphate or pentose phosphoketolase pathways. During the AP stage, when
 290 the community was in the exponential growth phase, pyruvate metabolism, especially the formate C-
 291 acetyltransferase, was expressed. In LAB capable of homofermentative metabolism, pyruvate
 292 formate lyase (PFL) is used during the transformation to mixed acid formation under glucose and
 293 oxygen limitation to increase the ATP yields (22). Here the beef was packaged under high oxygen
 294 MA and this enzyme is oxygen sensitive because it is cleaved and inhibited in the presence of oxygen
 295 (23). Nevertheless, the bacteria were found to express the *pfl* gene.

296
 297 The high oxygen MA and storage at cold (+6°C) temperatures created stress for the bacterial
 298 communities (Fig. 3). The cold shock genes were found active during the first days of shelf life before
 299 the communities reached the exponential growth phase. However, in case of *L. piscium* cold shock
 300 genes were still expressed during ES but this species was growing delayed in comparison to subsp.
 301 *gasicomitatum*. In the lag phase, and especially after the commercially deemed shelf life was over,
 302 oxygen induced stress responses increased and thus the microbial community members had to strive
 303 to cope with the high oxygen MA. Heme-dependent respiration is among the ways to cope with
 304 oxygen related stress and also the relative abundance of transcripts involved in respiration increased.
 305 Based on previous functional genomics studies with single species (10, 18, 24), we hypothesized that
 306 in addition to the rapid onset of fermentation, the heme-dependent respiration of leuconostocs may

307 play a major role for fitness under high oxygen MA also at the community level. Respiration has been
 308 shown to result in increased biomass of LAB, in several changes in the metabolism and in long-term
 309 survival (24-26). The increase in respiration with time indicates that oxygen stress becomes more
 310 evident for the community. Interestingly, respiration genes were found active in both bacterial and
 311 fungal members of the community in LS. Since the respiratory activity increased over time and was
 312 the highest in the AP and ES, the activity can be considered to play a role also in long-term survival
 313 and viability in a meat spoilage community during shelf life. In addition to the different cytochrome
 314 oxidases found in LAB, also ubiquinone Menaquinone-cytochrome c reductase complexes acting in
 315 electron donating and accepting reactions were active in the ES microbial community. In our recent
 316 coculture study (10), we noticed a similar trend in respect of subsp. *gasicomitatum*. It was the fastest-
 317 growing bacterium in the coculture containing *L. piscium* and *L. oligofermentans* (10). Subsp.
 318 *gasicomitatum* enhanced its nutrient-scavenging and growth capabilities in the presence of other LAB
 319 through upregulation of carbohydrate catabolic pathways, pyruvate fermentation enzymes, and
 320 ribosomal proteins (10). These findings are in line with the present study, highlighting the active role
 321 of this SSO in the community over the course of time. *D. aligida*, a psychrotrophic LAB isolated from
 322 vacuum packaged beef, has been isolated from various beef products (27) but was not abundant in
 323 the beef product studied (Table 1). The 80% O₂ clearly prevents growth of this bacteria and in a study
 324 where both high oxygen AMP and vacuum packaging were used, growth was higher in the latter (9).
 325 Thus, high oxygen MAP can be considered to support growth and activity of psychrotrophic LAB
 326 from the genera *Leuconostoc* and *Lactococcus*.

327

328 At the ES and LS stages, an increase in the activity of *noxE* gene was observed. As the microbes are
 329 growing actively while reaching the stationary phase, the use of carbohydrates leads to lack of
 330 potential electron acceptors. The *noxE* gene has been found to act in lowering the redox potential and
 331 thus enabling active metabolism (28). Based on the mapping, this gene was found active in *L. piscium*

332 and thus this species can lower the redox potential of its environment to create more optimal growth
333 conditions in MAP beef. The aldehyde-alcohol dehydrogenases were expressed at the ES and LS
334 stages. In *E. coli*, this gene (*adhE*) has been shown to have antioxidant activity (29). Also, the *adhE*
335 genes have been reported to possess moonlight functions such as oxidative stress protection (12, 29).
336
337 Gene *yngB* of *L. piscium*, encoding for fibronectin-binding protein, was actively transcribed in ES.
338 This gene has been associated with heat stress in *Lactococcus lactis* but here we found indications of
339 increased expression under oxidative stress on beef. In *L. piscium*, *yngB* (LACPI_1373, [(30)]) is a
340 fibronectin and collagen binding protein, and it belongs to a class of fibronectin binding proteins
341 without a signal peptide. Indications of the gene to be used both in fibronectin/collagen binding and
342 biofilm formation have been documented for *S. suis* ortholog (31) and *Bacillus subtilis* (32).
343
344 In ES, the sugar alcohol metabolism increased. The substrate for metabolism may originate from the
345 bacterial cell membranes since *Leuconostoc* and other LAB have genes for glycerophospholipid
346 metabolism. Another possibility is that polyols produced by the yeast community increased the sugar
347 alcohol metabolism (33).
348
349 In addition to carbohydrate metabolism and stress-induced reactions, we were able to capture novel
350 information on the amino acid metabolism during meat spoilage. The arginine deimination is of
351 interest since in *L. sakei* arginine deimination has been shown to increase the tolerance of both low
352 pH and acid stress (34). Agmatine can be produced from arginine by a broad range of other bacteria
353 through decarboxylation. The agmatine deiminase pathway, present e.g. in *L. piscium* (30), can help
354 bacteria in protection against environmental stresses, including low pH in a similar way to how the
355 arginine deiminase pathway does (35).
356

357 **Conclusions**

358 Since sensory changes do not follow bacterial concentrations linearly, more accurate approaches for
 359 detecting food spoilage are needed. Results from our experiment show that instead of measuring
 360 bacterial cell numbers for product quality evaluation, the focus should be on more detailed activity
 361 markers related to food spoilage. These markers occurring over the course of time might provide a
 362 useful way to evaluate product freshness. A shift toward a *Lactococcus* dominated community can be
 363 considered as one indication of the end of shelf life for the meat product as well as the appearance of
 364 yeasts. In addition, the expression of genes involved in cold shock reactions decreased closer to the
 365 end of shelf life. Also, the activity of genes involved in respiration and oxygen stress were found to
 366 become more pronounced when the product was spoiled. Unlike what was previously thought,
 367 carbohydrate metabolism was one of the most active functions throughout the time and in the change
 368 from fresh to spoiled product.

369 **Materials and Methods**

370 *Experiment description*

371 Twenty-two packages of tenderized beef loin fillets stored in MAP were bought in commercial
 372 packaging. The samples were collected from the same lot (comprises production of one day) of a
 373 large-scale producer. During the experiment, the samples were stored at +6°C. Two packages were
 374 sampled destructively each day for 11 days. Before opening, the concentrations of O₂ and CO₂ in the
 375 package gas were measured (Checkpoint, PBI Dansensor) through a double septum and, after
 376 opening, one fillet (approximately 120 g) from each package was immediately transferred to a
 377 stomacher bag with 10 ml of RNA later (Ambion) and 5 ml of peptone water. The bacterial cells were
 378 removed from the beef surface by stomacher (Stomacher 400; Seward, West Sussex, United
 379 Kingdom) at a low power for 30 s. The liquid was collected from the stomacher bag and cell collection
 380 was conducted via two-stage centrifugation to separate the mammalian and bacterial cells. First, the

381 supernatants were centrifuged in 15-ml conical tubes at 200 relative centrifugal force (RCF) for 3
382 min at +4°C to remove the fat and eukaryotic cells. The supernatant was collected and centrifuged in
383 1.5 ml Eppendorf tubes at 13000 rpm for 3 min at +4°C to collect the cells. The supernatant was
384 poured off and replaced with 500 μ l of RNAlater. The collected cells were stored at -70°C until
385 nucleic acid extraction.

386

387 Another fillet was used for quantitative microbiological and sensory analysis. Serial 10-fold dilution
388 series were made from 22 g of beef with 198 ml of peptone-salt buffer (0.1% peptone, 0.9% NaCl)
389 and homogenized by using a stomacher at medium power for 1 min, followed by cultivation onto
390 MRS plates (LAB). In addition, plate count agar (PCA) was used to quantify the total cultivable
391 microbes from the beef fillets. The PCA plates were incubated at 25°C for 3 to 5 days before colony
392 enumeration. The MRS plates were incubated in jars made anaerobic by a commercial atmosphere
393 generation system (AnaeroGen, Oxoid) at 25°C for 5 days, after which the CFU were calculated.
394 Sensory analyses were performed by a trained panel of at least five individuals. For these analyses,
395 the beef samples were equilibrated at room temperature. Beef samples from the same meat lot was
396 stored fresh (day 0) in the freezer (-20 °C) and used as a reference. The panelists evaluated the odor
397 and the appearance of the samples using a five-point scale (1 = severe defect, spoiled; 2 = clear defect,
398 spoiled; 3 = mild defect, satisfactory; 4 = good; 5 = excellent); the observed deficiencies were
399 described by the panelists.

400

401 *RNA extraction and library preparation*

402 The extraction protocol was modified from Chomczynski & Sacchi (36). First, the samples were
403 centrifuged at 16 000 RCF at +4°C for 10 min and the RNAlater was carefully removed. The pellet
404 was immediately covered with 500 μ l of Phenol-chloroform-isoamylalcohol (Sigma) and 500 μ l of
405 denaturation solution (4M guanidium thiocyanate, 24 mM sodium citrate, 0.5% sarcosyl and 0.1M

betamercaptoethanol). The samples were transferred to Lysing matrix E tubes (mBio) and beaten with beads in FastPrep-24 instrument (MP Biomedicals) for 30s at 5.5 m/s. After bead-beating, the tubes were placed on ice for 5 minutes followed by centrifugation at 13000 RCF at +4°C for 10 min. The supernatant was mixed with 500 μ l chloroform, vortexed and centrifuged at 4°C at 13000 RCF for 5 min. Nucleic acids were precipitated with 1:10 of 3M NaAc, 10 $\times$ volume of isopropanol and 1 μ l of GlycoBlue (Invitrogen) at -20°C for 2 hours. The nucleic acid pellet was washed with 70% ethanol and eluted to 100 μ l of DEPC water. DNA and RNA were purified with Qiagen Allprep DNA RNA kit according to the manufacturer's recommendations with additional DNase treatment. Resulting DNA and RNA quality was checked with gel electrophoresis and Agilent Bioanalyzer Nano chips, respectively. Concentration was checked with Cubit (Thermo).

The sequencing libraries were made both from the ribosomal RNA (rRNA) depleted libraries (mRNA library) and total RNA. rRNA depletion for the mRNA library was conducted with Ribozero kit (Epicentre) with the low input protocol. Illumina TruSeq® Stranded mRNA kit was used the library preparation. The samples were sequenced on an Illumina NextSeq at the DNA sequencing and genomics laboratory at the University of Helsinki.

Quality filtering

The raw reads were filtered for quality with cutadapt (37) to remove the reads with reverse adapter with quality cutoff Q20 and with a length below 60 bp. The quality filtered reads were mapped to *Bos taurus* genome (accession number NKLS000000000.2) using Bowtie2 (38) with default parameters and reads mapping to *B. taurus* were discarded.

429 *rRNA mapping*

430 The rRNA reads from the quality filtered non-*B. taurus* reads of the total RNA libraries were searched
431 with Metaxa2 (39) and blasted against Silva 138 database (40) to identify the 16S rRNA reads and
432 thus analyze the change in active species composition of the samples.

433

434 *Annotation*

435 All sequence reads from mRNA libraries were annotated against KEGG (41) and SEED (42)
436 databases with e-value cutoff 1e-5, minimum identity cutoff 60% and min alignment cutoff 15 bp at
437 MG-RAST (43). The annotated genes were normalized by sample read number. Three bacterial
438 genomes *gelidum* subs. *gasicomitatum* (LMG 18811, FN822744.1), *L. piscium* (MKFS47,
439 NZ_LN774769) and *D. algida* (DSM 15638, NZ_AZDI01000000) were selected for more thorough
440 analysis. The sequence reads from all samples were mapped to the Uniprot (44) genes of each genome
441 with Bowtie2 (38). In addition, the reads were annotated to species in Metaphlann2 (14) and specific
442 pathways examined in Humann2 (45).

443

444 *Statistics*

445 Bray-Curtis distance matrix from relative abundance normalized 16S rRNA picked from the total
446 RNA libraries and metabolic pathway data from the mRNA libraries were ordinated with NMSD in
447 R (46) using vegan (47), ggplot (48) and Phyloseq (49). Bacterial abundances were matched with the
448 factor “Sample stage” with the function envfit from the Vegan package and 80% confidence area was
449 used for standard deviation of the centroids. The statistical differences among the functional profiles
450 of the three time points were analyzed with STAMP software package (50). An ANOVA analysis
451 with Games-Howell post-hoc test and Storey’s FDR for correction was conducted. Sequence data
452 were deposited at the European Nucleotide Archive (ENA) under the project PRJEB20288. The
453 annotated metagenomes are available at MG-RAST under project BEEF_RNA.

454 **Acknowledgements**

455 We thank Ms. Henna Niinivirta for skillful technical assistance. The CSC - IT Center for Science Ltd
456 is acknowledged for providing computational resources. The project was financially supported by the
457 Academy of Finland CODELAB (307855) and a grant to JH from the Walter Ehrström Foundation.

458 **References**

- 459 1. **Doulgeraki AI, Ercolini D, Villani F, Nychas GJ.** 2012. Spoilage microbiota associated to the
460 storage of raw meat in different conditions. *Int J Food Microbiol* **157**:130-141.
- 461 2. **Pothakos V, Devlieghere F, Villani F, Björkroth J, Ercolini D.** 2015. Lactic acid bacteria and
462 their controversial role in fresh meat spoilage. *Meat Sci* **109**:66-74.
- 463 3. **Moretro T, Moen B, Heir E, Hansen AA, Langsrud S.** 2016. Contamination of salmon fillets
464 and processing plants with spoilage bacteria. *Int J Food Microbiol* **237**:98-108.
- 465 4. **Mann E, Wetzels SU, Pinior B, Metzler-Zebeli BU, Wagner M, Schmitz-Esser S.** 2016.
466 Psychrophile spoilers dominate the bacterial microbiome in musculature samples of slaughter pigs.
467 *Meat Sci* **117**:36-40.
- 468 5. **Pothakos V, Stellato G, Ercolini D, Devlieghere F.** 2015. Processing environment and
469 ingredients are both sources of *Leuconostoc gelidum*, which emerges as major spoiler in ready-to-
470 eat meals. *Appl Environ Microbiol* **81**:3529-41.
- 471 6. **De Filippis F, La Stora A, Villani F, Ercolini D.** 2013. Exploring the sources of bacterial
472 spoilers in beefsteaks by culture-independent high-throughput sequencing. *PLoS One* **8**:e70222.
- 473 7. **Hultman J, Rahkila R, Ali J, Rousu J, Björkroth KJ.** 2015. Meat Processing Plant
474 Microbiome and Contamination Patterns of Cold-Tolerant Bacteria Causing Food Safety and

475 Spoilage Risks in the Manufacture of Vacuum-Packaged Cooked Sausages. *Appl Environ*
476 *Microbiol* **81**:7088-7097.

477 8. **Säde E, Penttinen K, Björkroth J, Hultman J.** 2017. Exploring lot-to-lot variation in spoilage
478 bacterial communities on commercial modified atmosphere packaged beef. *Food Microbiol* **62**:147-
479 152.

480 9. **Jääskeläinen E, Hultman J, Parshintsev J, Riekkola ML, Björkroth J.** 2016. Development of
481 spoilage bacterial community and volatile compounds in chilled beef under vacuum or high oxygen
482 atmospheres. *Int J Food Microbiol* **223**:25-32.

483 10. **Andreevskaya M, Jääskeläinen E, Johansson P, Ylinen A, Paulin L, Björkroth J, Auvinen**
484 **P.** 2018. Food Spoilage-Associated *Leuconostoc*, *Lactococcus*, and *Lactobacillus* Species Display
485 Different Survival Strategies in Response to Competition. *Appl Environ Microbiol* **84**:e00554-18

486 11. **Vido K, Diemer H, Van Dorselaer A, Leize E, Juillard V, Gruss A, Gaudu P.** 2005. Roles
487 of thioredoxin reductase during the aerobic life of *Lactococcus lactis*. *J Bacteriol* **187**:601-610.

488 12. **Yang W, Li E, Kairong T, Stanley SL, Jr.** 1994. Entamoeba histolytica has an alcohol
489 dehydrogenase homologous to the multifunctional adhE gene product of *Escherichia coli*. *Mol*
490 *Biochem Parasitol* **64**:253-260.

491 13. **Benaroudj N, Lee DH, Goldberg AL.** 2001. Trehalose accumulation during cellular stress
492 protects cells and cellular proteins from damage by oxygen radicals. *J Biol Chem* **276**:24261-
493 24267.

494 14. **Truong DT, Franzosa EA, Tickle TL, Scholz M, Weingart G, Pasolli E, Tett A,**
495 **Huttenhower C, Segata N.** 2015. MetaPhlAn2 for enhanced metagenomic taxonomic profiling.
496 *Nat Methods* **12**:902-903.

- 497 15. **Korkeala H, Alanko T, Mäkelä P, Lindroth S.** 1989. Shelf-life of vacuum-packed cooked
498 ring sausages at different chill temperatures. *Int J Food Microbiol* **9**:237-247.
- 499 16. **Nieminen TT, Nummela M, Björkroth J.** 2015. Packaging gas selects lactic acid bacterial
500 communities on raw pork. *J Appl Microbiol* **119**:1310-1316.
- 501 17. **Pothakos V, Snauwaert C, De Vos P, Huys G, Devlieghere F.** 2014. Monitoring
502 psychrotrophic lactic acid bacteria contamination in a ready-to-eat vegetable salad production
503 environment. *Int J Food Microbiol* **185**:7-16.
- 504 18. **Johansson P, Paulin L, Säde E, Salovuori N, Alatalo ER, Björkroth KJ, Auvinen P.** 2011.
505 Genome sequence of a food spoilage lactic acid bacterium, *Leuconostoc gasicomitatum* LMG
506 18811T, in association with specific spoilage reactions. *Appl Environ Microbiol* **77**:4344-4351.
- 507 19. **Vihavainen EJ, Björkroth KJ.** 2009. Diversity of *Leuconostoc gasicomitatum* associated with
508 meat spoilage. *Int J Food Microbiol* **136**:32-36.
- 509 20. **Coppola S, Mauriello G, Aponte M, Moschetti G, Villani F.** 2000. Microbial succession
510 during ripening of Naples-type salami, a southern Italian fermented sausage. *Meat Sci* **56**:321-329.
- 511 21. **Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman**
512 **DA, Fraser-Liggett CM, Nelson KE.** 2006. Metagenomic analysis of the human distal gut
513 microbiome. *Science* **312**:1355-1359.
- 514 22. **Wagner N, Tran QH, Richter H, Selzer PM, Uden G.** 2005. Pyruvate fermentation by
515 *Oenococcus oeni* and *Leuconostoc mesenteroides* and role of pyruvate dehydrogenase in anaerobic
516 fermentation. *Appl Environ Microbiol* **71**:4966-4971.
- 517 23. **Reddy SG, Wong KK, Parast CV, Peisach J, Magliozzo RS, Kozarich JW.** 1998. Dioxygen
518 inactivation of pyruvate formate-lyase: EPR evidence for the formation of protein-based sulfinyl
519 and peroxy radicals. *Biochemistry* **37**:558-563.

- 520 24. **Jääskeläinen E, Johansson P, Kostiaainen O, Nieminen T, Schmidt G, Somervuo P,**
521 **Mohsina M, Vanninen P, Auvinen P, Björkroth J.** 2013. Significance of Heme-based
522 Respiration in Meat Spoilage caused by *Leuconostoc gasicomitatum*. Appl Environ Microbiol
523 **79**:1078-1085.
- 524 25. **Pedersen MB, Gaudu P, Lechardeur D, Petit MA, Gruss A.** 2012. Aerobic respiration
525 metabolism in lactic acid bacteria and uses in biotechnology. Annu Rev Food Sci Technol **3**:37-58.
- 526 26. **Lechardeur D, Cesselin B, Fernandez A, Lamberet G, Garrigues C, Pedersen M, Gaudu**
527 **P, Gruss A.** 2011. Using heme as an energy boost for lactic acid bacteria. Curr Opin Biotechnol
528 **22**:143-149.
- 529 27. **Kato Y, Sakala RM, Hayashidani H, Kiuchi A, Kaneuchi C, Ogawa M.** 2000. *Lactobacillus*
530 *algidus* sp. nov., a psychrophilic lactic acid bacterium isolated from vacuum-packaged refrigerated
531 beef. Int J Syst Evol Microbiol **50 Pt 3**:1143-1149.
- 532 28. **Tachon S, Brandsma JB, Yvon M.** 2010. NoxE NADH oxidase and the electron transport
533 chain are responsible for the ability of *Lactococcus lactis* to decrease the redox potential of milk.
534 Appl Environ Microbiol **76**:1311-1319.
- 535 29. **Echave P, Tamarit J, Cabiscol E, Ros J.** 2003. Novel antioxidant role of alcohol
536 dehydrogenase E from Escherichia coli. J Biol Chem **278**:30193-30198.
- 537 30. **Andreevskaya M, Johansson P, Laine P, Smolander OP, Sonck M, Rahkila R,**
538 **Jääskeläinen E, Paulin L, Auvinen P, Björkroth J.** 2015. Genome Sequence and Transcriptome
539 Analysis of Meat-Spoilage-Associated Lactic Acid Bacterium *Lactococcus piscium* MKFS47. Appl
540 Environ Microbiol **81**:3800-3811.
- 541 31. **Musyoki AM, Shi Z, Xuan C, Lu G, Qi J, Gao F, Zheng B, Zhang Q, Li Y, Haywood J, Liu**
542 **C, Yan J, Shi Y, Gao GF.** 2016. Structural and functional analysis of an anchorless fibronectin-

543 binding protein FBPS from Gram-positive bacterium *Streptococcus suis*. Proc Natl Acad Sci U S A
544 **113**:13869-13874.

545 32. **Rodriguez Ayala F, Bauman C, Bartolini M, Saball E, Salvarrey M, Lenini C, Cogliati S,**
546 **Strauch M, Grau R.** 2017. Transcriptional regulation of adhesive properties of *Bacillus subtilis* to
547 extracellular matrix proteins through the fibronectin-binding protein YloA. Mol Microbiol **104**:804-
548 821.

549 33. **Shen B, Hohmann S, Jensen RG, Bohnert a.** 1999. Roles of sugar alcohols in osmotic stress
550 adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. Plant Physiol **121**:45-52.

551 34. **Champomier Verges MC, Zuniga M, Morel-Deville F, Perez-Martinez G, Zagorec M,**
552 **Ehrlich SD.** 1999. Relationships between arginine degradation, pH and survival in *Lactobacillus*
553 *sakei*. FEMS Microbiol Lett **180**:297-304.

554 35. **Griswold AR, Jameson-Lee M, Burne RA.** 2006. Regulation and physiologic significance of
555 the agmatine deiminase system of *Streptococcus mutans* UA159. J Bacteriol **188**:834-841.

556 36. **Chomczynski P, Sacchi N.** 2006. The single-step method of RNA isolation by acid
557 guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. Nat Protoc
558 **1**:581-585.

559 37. **Martin M.** 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads.
560 EMBnet.journal **17**.

561 38. **Langmead B, Salzberg SL.** 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods
562 **9**:357-359.

563 39. **Bengtsson-Palme J, Richardson RT, Meola M, Wurzbacher C, Tremblay ED, Thorell K,**
564 **Kanger K, Eriksson KM, Bilodeau GJ, Johnson RM, Hartmann M, Nilsson RH.** 2018.

565 Metaxa2 Database Builder: enabling taxonomic identification from metagenomic or metabarcoding
566 data using any genetic marker. *Bioinformatics* **34**:4027-4033.

567 40. **Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glockner FO.**
568 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based
569 tools. *Nucleic Acids Res* **41**:D590-6.

570 41. **Kanehisa M, Goto S.** 2000. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids*
571 *Res* **28**:27-30.

572 42. **Overbeek R, Begley T, Butler RM, Choudhuri JV, Chuang HY, Cohoon M, de Crecy-**
573 **Lagard V, Diaz N, Disz T, Edwards R, Fonstein M, Frank ED, Gerdes S, Glass EM,**
574 **Goesmann A, Hanson A, Iwata-Reuyl D, Jensen R, Jamshidi N, Krause L, Kubal M, Larsen**
575 **N, Linke B, McHardy AC, Meyer F, Neuweiger H, Olsen G, Olson R, Osterman A, Portnoy V,**
576 **Pusch GD, Rodionov DA, Ruckert C, Steiner J, Stevens R, Thiele I, Vassieva O, Ye Y,**
577 **Zagnitko O, Vonstein V.** 2005. The subsystems approach to genome annotation and its use in the
578 project to annotate 1000 genomes. *Nucleic Acids Res* **33**:5691-5702.

579 43. **Keegan KP, Glass EM, Meyer F.** 2016. MG-RAST, a Metagenomics Service for Analysis of
580 Microbial Community Structure and Function. *Methods Mol Biol* **1399**:207-233.

581 44. **UniProt Consortium.** 2019. UniProt: a worldwide hub of protein knowledge. *Nucleic Acids*
582 *Res* **47**:D506-D515.

583 45. **Franzosa EA, McIver LJ, Rahnavard G, Thompson LR, Schirmer M, Weingart G, Lipson**
584 **KS, Knight R, Caporaso JG, Segata N, Huttenhower C.** 2018. Species-level functional profiling
585 of metagenomes and metatranscriptomes. *Nat Methods* **15**:962-968.

586 46. **R Core Team** (2019). R: A language and environment for statistical computing. R Foundation
587 for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.

588 47. **Oksanen J, Blanchet GF, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR,**
589 **O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E and Wagner H** (2019). **vegan:**
590 **Community Ecology Package.** R package version 2.5-5. [https://CRAN.R-](https://CRAN.R-project.org/package=vegan)
591 [project.org/package=vegan](https://CRAN.R-project.org/package=vegan)

592 48. **Wickham H.** 2016. **ggplot2: Elegant Graphics for Data Analysis.** Springer-Verlag, New York.
593 <https://ggplot2.tidyverse.org>.

594 49. **McMurdie PJ, Holmes S.** 2013. **phyloseq: an R package for reproducible interactive analysis**
595 **and graphics of microbiome census data.** PLoS One **8**:e61217.

596 50. **Parks DH, Beiko RG.** 2010. **Identifying biologically relevant differences between**
597 **metagenomic communities.** Bioinformatics **26**:715-721.

598

599 **Table 1 legend**

600 **Table 1.** Percentage of trimmed sequence reads from the mRNA fraction mapping to the genomes
 601 of three selected specific spoilage bacteria *Leuconostoc gelidum* subsp. *gasicomitatum*
 602 (LMG18811^r), *Lactococcus piscium* (MKFS37) and *Dellagليا algida* (DSM 15638^r). Two
 603 packages were opened for each point of analysis.

604

605 **Legends to the figures**

606 **Figure 1.** Bacterial concentration (PCA: plate count agar, total bacterial counts; MRS: de Mann
 607 Rogosa Sharpe, LAB counts) and main sensory findings during experiment. The three stages related
 608 to the product were nominated as acceptable product (AP, days 3-5), early spoilage (ES, days 6-9),
 609 and late spoilage stage (LS, days 10-11). Curve presents mean value obtained from analyses of two
 610 packages, whiskers are related to each value obtained.

611

612 **Figure 2.** A) Assignment of 16S rRNA reads to taxa using Silva 138 database. The results of the
 613 most abundant families are shown. B) Change in activity during the experiment based on read
 614 annotation against SEED subsystems. C) Clustering of samples from different time points in
 615 nonmetric multidimensional scaling (NMDS) plot with 16S rRNA gene assignment and D) by the
 616 SEED database annotations. Samples from day 1 were discarded from the analysis due to the low
 617 number of reads recovered from sequencing. The samples were clustered into acceptable product
 618 (AP), early spoilage (ES) and late spoilage (LS). Blue lines show the labeled group centroids of the
 619 samples and the circle around the centroids is the 80% confidence area for standard deviation of the
 620 centroids.

621 **Figure 3.** Relative abundance of transcripts involved in different SEED subsystem A) Cold shock
 622 B) Oxidative stress C) Respiration categories in AP, ES and LS with statistically differing
 623 abundances. Denote difference on y-axis in each of the plots. The median value is shown as line
 624 within the box and the mean value as a star.

625

626 **Figure 4.** Carbohydrate metabolism activities of selected main pathways. Relative abundance of
 627 genes in pathways involved in carbohydrate metabolism in acceptable product (AP), early spoilage
 628 (ES) and late spoilage (LS) are denoted with the bubbles with sphere size relative to the abundance
 629 of transcripts. Pie charts visualize the abundance of expressed genes in each stage and the difference
 630 in gene transcript abundance. Most abundant genes of each pathway are shown and organized in
 631 clockwise order.

632

633 **Figure 5.** Proportion of reads belonging to ascomycotal family Debaryomycetaceae based on
 634 Humann2 annotation.

635

636 **Figure 6.** Most active functions and phyla in three stages, i.e. acceptable product, early spoilage and
 637 late spoilage, of a meat spoilage community developing in a high-oxygen modified atmosphere
 638 packaged beef product.

639

Table 1. Percentage of trimmed sequence reads from the mRNA fraction mapping to the genomes of three selected specific spoilage bacteria *Leuconostoc gelidum* subsp. *gasicomitatum* (LMG18811^T), *Lactococcus piscium* (MKFS37) and *Dellaglia algida* (DSM 15638^T). Two packages were opened for each point of analysis.

Sample	<i>L. gelidum</i> subsp. <i>gasicomitatum</i>	<i>L. piscium</i>	<i>D. algida</i>	Sum
Day2-1	3.2	0.1	1.1	4.4
Day2-2	2.5	0.1	0.5	3.1
Day3-1	19.7	0.7	7.1	27.5
Day3-2	30.0	0.8	5.9	36.7
Day4-1	27.7	3.9	7.0	38.6
Day4-2	40.7	1.3	19.9	61.9
Day5-1	43.7	1.7	6.4	51.8
Day5-2	49.4	6.1	7.8	63,3
Day6-1	39.7	5.9	4.8	50.4
Day6-2	27.9	6.5	3.1	37.5
Day7-1	20.5	17.4	4.2	42.1
Day7-2	25.4	10.9	2.8	39.1
Day8-1	37.8	10.4	5.4	53.6
Day8-2	51.0	7.9	5.1	64.0
Day9-1	15.5	8.7	4.0	28.2
Day9-2	32.7	7.9	4.5	45.1
Day10-1	13.2	2.4	1.7	17.3
Day10-2	15.9	7.0	2.4	25.3
Day11-1	18.6	4.0	1.4	24.0
Day11-2	8.0	4.8	1.7	14.5

Figure 1. Bacterial concentrations (Plate count agar (PCA), total bacterial counts; de Mann Rogosa Sharpe agar (MRS), lactic acid bacterium counts) and main sensory findings during the experiment. The three stages related to sensory quality scores of the product were nominated as acceptable product (AP, days 3-5), early spoilage (ES, days 6-9), and late spoilage (LS, days 10-11) stages. Curve drawn from the mean values obtained from the results of two packages opened each day, whisker edges display concentration of each package.

Figure 2. A) Assignment of 16S rRNA reads to taxa using Silva 138 database. The results of the most abundant families are shown. B) Change in activity during the experiment based on read annotation against SEED subsystems. C) Clustering of samples from different time points in nonmetric multidimensional scaling (NMDS) plot with 16S rRNA gene assignment and D) by the SEED database annotations. Samples from day 1 were discarded from the analysis due to the low number of reads recovered from sequencing. The samples were clustered into acceptable product (AP), early spoilage (ES) and late spoilage (LS). Blue lines show the labeled group centroids of the samples and the circle around the centroids is the 80% confidence area for standard deviation of the centroids.

Figure 3. Relative abundance of transcripts involved in different SEED subsystem A) Cold shock B) Oxidative stress C) Respiration categories in AP, ES and LS with statistically differing abundances. Denote difference on y-axis in each of the plots. The median value is shown as line within the box and the mean value as a star.

Figure 4. Carbohydrate metabolism activities of selected main pathways. Relative abundance of genes in pathways involved in carbohydrate metabolism in acceptable product (AP), early spoilage (ES) and late spoilage (LS) are denoted with the bubbles with sphere size relative to the abundance of transcripts. Pie charts visualize the abundance of expressed genes in each stage and the difference in gene transcript abundance. Most abundant genes of each pathway are shown and organized in clockwise order.

Figure 5. Proportion of reads belonging to ascomycotal family Debaryomycetaceae based on Humann2 annotation.

Figure 6. Most active functions and phyla in three stages, i.e. acceptable product, early spoilage and late spoilage, of a meat spoilage community developing in a high-oxygen modified atmosphere packaged beef product.