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Deciphering the Upper Respiratory Tract Microbiota Complexity in Healthy Calves and Calves Developing Respiratory Disease Using Shotgun Metagenomics

Journal:	*Journal of Dairy Science*
Manuscript ID	JDS-16-11522.R3
Article Type:	Research
Date Submitted by the Author:	19-Oct-2016
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Key Words:	Bovine Respiratory Disease, Whole Genome Sequencing, Calf

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1 **Interpretive summary: Metagenomics of bovine respiratory disease**

2 *Gaeta N. et al.*

3 We evaluated the microbiota of the upper respiratory tract of healthy Holstein calves and
4 calves that developed bovine respiratory disease using shotgun metagenomics. Deep
5 nasopharyngeal swabs were performed on 16 Holstein heifer calves at 14 and 28 days of life.
6 Samples were dominated by five predominant phyla: *Proteobacteria, Firmicutes, Actinobacteria,*
7 *Bacteroidetes* and *Tenericutes.* At the genus level, differences between groups were observed for
8 *Pseudomonas* spp. *Moraxella catarrhalis* was detected in all groups of samples. The resistance to
9 antibiotics and toxic compounds profiling analysis revealed differences in cobalt-zinc-cadmium
10 resistance especially between BRD and healthy calves at their 14th day of life.

11 **METAGENOMICS OF BOVINE RESPIRATORY DISEASE**

12 **Deciphering the Upper Respiratory Tract Microbiota Complexity in Healthy Calves and**
13 **Calves Developing Respiratory Disease Using Shotgun Metagenomics**

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25 **ABSTRACT**

26 Bovine Respiratory Disease (BRD) is a multi-factorial disorder that remains responsible for
27 severe economic losses within dairy and feedlot herds. Advances in next generation sequencing
28 allow the characterization of microbial communities present in clinical samples, including non-
29 culturable bacteria. Our aim here was to evaluate the microbiota of the upper respiratory tract of
30 healthy calves and calves developing BRD using whole genome sequencing (shotgun
31 metagenomics). Deep nasopharyngeal swabs were performed on sixteen Holstein heifer calves (ten
32 healthy and six that were diagnosed with BRD during the study) at 14 and 28 days of life in one
33 dairy herd located near Ithaca, New York. Total DNA was extracted and whole genome sequencing
34 was performed using the MiSeq Illumina platform. Samples were dominated by five predominant
35 phyla: *Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes* and *Tenericutes.* At the genus
36 level, differences between groups were observed for *Pseudomonas* spp. At the species level,
37 *Mannheimia haemolytica* was the most abundant bacterium detected. Significant differences
38 between different groups of calves were detected in the relative abundance of *Pseudomonas*
39 *fluorescens. Pasteurella multocida* was amongst the 20 most abundant species. Additionally,
40 *Moraxella catarrhalis,* commonly associated with pneumonia in humans, was detected in all groups
41 of calves. The resistance to antibiotics and compounds profiling analysis revealed differences in
42 cobalt-zinc-cadmium resistance. Further research that will elucidate the role of *Moraxella*
43 *catarrhalis* in BRD is warranted. Cobalt-Zinc-Cadmium resistant genes observed mostly in BRD
44 calves might be associated with difficulties in the treatment using antibiotics.

45 **Key words:** Bovine Respiratory Disease, Whole Genome Sequencing, Calf

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48 **INTRODUCTION**

49 Bovine Respiratory Disease (**BRD**) is a multi-factorial disorder (Gershwin et al., 2015) that
50 remains responsible for severe economic losses in dairy and feedlot herds, due to its high morbidity
51 and mortality rates (USDA, 2013). Furthermore, BRD has substantial long-term consequences on
52 performance by negatively impacting growth, reproductive performance, and longevity (Waltner-
53 Toews et al., 1986; Virtala et al., 1996a,b; Cernicchiaro et al., 2013). It has been estimated that 7%
54 of the total production cost in feedlots in the United States is related to BRD (Griffin, 1997).

55 The upper respiratory tract of animals normally harbors a variety of microorganisms that
56 coexist in a harmonious state (Bosch et al., 2013). However, certain events can lead to imbalances
57 in this microbiome and eventually to respiratory disease. In cattle, viral infections, stress (i.e.
58 transportation and mixing), and environmental conditions (i.e. stocking density, climate, and
59 temperature) are important predisposing factors to this imbalance, leading to respiratory tract
60 bacterial infections facilitated by the host's immune suppression (Griffin et al., 2010). The most
61 commonly described bacteria in BRD are *Mannheimia haemolytica*, *Pasteurella multocida,*
62 *Histophilus somni* and *Mycoplasma* spp., particularly *Mycoplasma bovis* (Aebi et al., 2015; Holman
63 et al., 2015a). These species are considered part of the upper respiratory tract microbiome.
64 Similarly, humans' upper respiratory tract serves as a reservoir of commensal bacteria such as
65 *Haemophilus influenza, Streptococcus pneumoniae, Staphylococcus aureus,* and *Moraxella*
66 *catarrhalis* which can in certain cases invade the lungs and cause pneumonia (Bosch et al., 2013).

67 Culture-dependent techniques are often used as a diagnostic method to determine
68 microorganisms associated with respiratory disease. However, certain limitations are associated
69 with these techniques, the main one being that several important microbes are difficult if not
70 impossible to culture in the laboratory (Sharpton, 2014). Advances in next generation sequencing
71 have allowed in depth investigation of clinical samples' microbiomes, determining its taxonomic

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72 composition including unculturable species. Whole genome sequencing (shotgun metagenomics),
73 differently from 16S metataxonomics, provides with taxonomic descriptions of microbial
74 communities, but at the same time creates important information regarding gene prediction, protein
75 diversity and functional profile (Sharpton, 2014).

76 Studies using 16S metataxonomics for the evaluation of the nasal, nasopharyngeal and lung
77 microbiota in feedlot cattle have already been published (Holman et al., 2015b; Timsit et al., 2016).
78 However, and to the best of our knowledge, shotgun metagenomics has not been used yet to
79 investigate BRD. Therefore, our aim was to characterize the microbiota of the upper respiratory
80 tract of healthy calves and calves developing BRD using whole genome sequencing, and identify
81 bacteria that may be related to the development of BRD and describe their functional profile.

82

83 **MATERIAL AND METHODS**

84 The present study was conducted at the Department of Population Medicine and Diagnostic
85 Science, College of Veterinary Medicine, Cornell University, New York, from August 2015 until
86 January 2016. Sixteen Holstein heifer calves were selected from a larger cohort of 174 animals that
87 were included in a different experiment (Lima et al., 2016). This larger scale study was conducted
88 on a commercial dairy farm located near Ithaca, New York that at the time of the study had around
89 7000 animals in total (3,500 lactating Holsteins cows), and approximately 600 pre-weaning calves
90 that were raised in groups of 25 animals per pen. All procedures were carried out in agreement with
91 the guidelines of the Institutional Animal Care and use Committee of Cornell University (Protocol
92 number: 2013-0076).

93 ***Animals and facilities***

94 Pregnant cows at stage 1 or 2 of parturition were transferred from the close-up free-stall barn
95 into two maternity pens (400 m^2 deep-bedded pens). After parturition, calves were removed from

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96 the maternity pen and placed into a newborn pen bedded with dry sawdust and heated with heating
97 lamps during the winter months. Feeding and pen characteristics were described by Lima et al.,
98 (2016). Calves used in this study were allocated in 9 different pens (25 calves per pen) and all
99 calves remained in the same pen from day one of life until fully weaned (approximately 65 days of
100 life). The age range of calves within a pen was not greater than 3 days.

101 ***Case definition***

102 BRD was initially defined when two or more of the following clinical signs were detected in
103 a calf: cough, rectal temperature > 39.5 °C, respiratory rate > 40 breaths/min, increased
104 cranioventral lung sounds or wheezes. Two dedicated farm employees with over 10 years of
105 experience and trained by Cornell University veterinarians (Ambulatory and Production Medicine
106 Department), were responsible for overseeing the calf facility and making the initial detection of
107 BRD. An experienced veterinarian member of our research team performed a second confirmatory
108 physical examination. Calves diagnosed with BRD were treated according to standard farm protocol
109 (Resflor Gold, Merck Animal Health). Treatment was administered at the day of the diagnosis.

110 ***Deep nasopharyngeal swab collection***

111 As part of this different larger scale observational study deep nasopharyngeal (DNP) swabs
112 were collected from a cohort of 174 Holstein heifer calves on days 3, 14, 28, and 35 of their life
113 using a 20-cm DNA-free sterile swab (Puritan Medical Products, Guilford, ME) covered by a thin
114 sterile plastic sheath, as described by Lima et al. (2016). Samples were kept on ice until they were
115 transferred to the laboratory and then stored at -20 °C until further processing. Deep nasopharyngeal
116 swabs collected at 14 and 28 days of life from 10 calves that remained healthy throughout the pre-
117 weaning period (randomly selected among all the calves that remained healthy throughout the
118 study) and 6 calves that showed clinical signs of BRD before weaning (randomly selected among
119 all the calves that showed signs of BRD during the study) were used for the present study.

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120 Bovine respiratory disease was on average diagnosed on the 19th day of the calves' life. The
121 earliest day of diagnosis was the 14th with the latest being the 26th. We therefore had four groups of
122 samples: 10 samples obtained from healthy calves at 14 days of life (healthy-14d); 6 samples
123 obtained at 14 days of life from calves that developed BRD between 14 and 26 days of life (BRD-
124 14d); 10 samples obtained from healthy calves at 28 days of life (healthy-28d); and 6 samples
125 obtained at 28 days of life from calves that developed BRD between 14 and 26 days of life (BRD-
126 28d).

127 ***DNA extraction and Whole Genome Sequencing***

128 Total DNA was extracted from DNP samples by adding 1.5 mL of DNA-free water into
129 microcentrifuge tube containing cotton swabs, followed by mechanic disruption using a Mini-
130 Beader-8 (Biospec Products, Battersville, OK). Swabs were removed from the tubes and the
131 remaining liquid was centrifuged at 13,000 x g for 10 minutes at room temperature. The DNA was
132 extracted from pellets using PowerSoil DNA Isolation Kit (MO BIO Laboratory Inc., Carlsbad, CA)
133 according to the manufacturer's protocol (Lima et al., 2016).

134 Samples were diluted by adding UltraPure Water (Invitrogen, Waltham, MA) until a
135 concentration of 2.5ng/µL was reached, measured using Qubit (Thermo Fisher Scientific, Waltham,
136 MA) fluorometer. Sample library was prepared using Nextera DNA Sample Preparation Kit
137 (Illumina Inc. San Diego, CA) according to the manufacturer's protocol.

138 The library for cluster generation and sequencing was prepared according to Illumina's
139 protocol. Briefly, a mixture of 5 µL DNA (4nM) and 0.2 N NaOH was vortexed and centrifuged to
140 280 x g for 1 minute. The mixture was incubated for 5 minutes to allow DNA denaturation.
141 Following DNA denaturation, 990 µL of Hybridization Buffer were added to dilute DNA and
142 obtain a 2 nM library. Quality control for cluster generation, sequencing and alignment was
143 provided by adding the PhiX Control - 4 nM (Illumina Inc. San Diego, CA), prepared using 2 µL of

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144 10 nM PhiX library and 3 µL of 10 NM Tris-Cl pH8.5 with 0,1% Tween 20. Pair-end sequencing
145 was performed using a MiSeq Reagent Kit v3 (600-cycles) in the Illumina MiSeq platform.

146 ***Bioinformatics and statistical analysis***

147 Raw data files were de-multiplexed and converted to fastq files using Casava v.1.8.2 (Illumina,
148 Inc, San Diego, CA). Fastq files were concatenated and uploaded to the Metagenome Rapid
149 Annotation using Subsystem Technology (**MG-RAST**) server to determine relative abundance of
150 the microbiota at the phylum, genus and species levels, and metabolic characteristics prediction. In
151 the MG-RAST pipeline, sequences were subjected to quality control, including dereplication
152 (removal of sequences produced by sequencing artifacts), removal of host-specific species
153 sequences (Bos taurus, UMD v3.0), ambiguous base filtering (removal of sequences with >5
154 ambiguous base pairs) and a length filtering (removal of sequences with a length >2 standard
155 deviations from the mean). To reduce the impact of experimental noise/error, the normalized data
156 option of MG- RAST was used. Normalization was performed by a log2-based transformation (log2
157 (x + 1)) followed by standardization within each sample and linear scaling (across all samples).
158 Non-redundant multi-source protein annotation database (M5NR) was the annotation source used
159 for organism classification. Organism abundance was analyzed using a "Best Hit Classification"
160 approach with a maximum e-value of 1 × 10-5, minimum identity cutoff of 60%, and a minimum
161 alignment length cut-off of 15. The SEED was the annotation source used for predicted metabolic
162 profiles. The predicted metabolic abundance analysis was performed using "Hierarchical
163 Classification", with a maximum e-value of 1 × 10-5, minimum identity of 60%, and a minimum
164 alignment length cut-off of 15.

165 Normalized abundances of phyla, genera, species and functional profile elements were
166 extracted from MG-RAST and analyzed using ANOVA in conjunction with Tukey test for multiple
167 comparisons using JMP Pro 12 (SAS Institute Inc., NC). Heatmap for phyla distribution were
168 constructed in JMP Pro 12. Results are presented as least square means followed by the standard

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169 error of the mean. Dependent variables in our statistical analyses were the normalized relative
170 abundances of microbial types with the independent variable being the different health status and
171 sampling time (BRD -14d; BRD-28d; Healthy-14d; Healthy-28d). Comparisons were performed in
172 order to identify potential bacteria and functional features that may be related to the development of
173 BRD during the period that calves are more predisposed to it (Virtala et al., 1996). Variables with P
174 < 0.05 were considered statistically different. Variables with $0.1 < P < 0.05$ were considered as
175 statistical tendencies.

176 Shotgun sequencing data have been deposited in MG-RAST under the following accession
177 numbers (4678805.3, 4678806.3, 4678807.3, 4678808.3, 4678809.3, 4678810.3, 4678811.3,
178 4678812.3, 4678813.3, 4678814.3, 4678815.3, 4678816.3, 4678817.3, 4678818.3, 4678819.3,
179 4678820.3, 4678821.3, 4678822.3, 4678823.3, 4678824.3, 4678825.3, 4678826.3, 4678827.3,
180 4678828.3, 4678829.3, 4678830.3, 4678831.3, 4678832.3, 4678833.3, 4678834.3, 4678835.3,
181 4678836.3).

182

183 **RESULTS**

184 ***Sequencing results***

185 Sequencing of DNP samples generated 21,155,233 sequences (mean number of sequences
186 per sample was: 661,101; median: 638,404; range = 383,190 - 994,172); 2,307,911,887 basepairs
187 (mean: 72,122,246.46; median: 73,160,664; range = 40,628-100,847,895); 310,656 high quality
188 sequences (mean: 9,708; median: 9,330; range = 4,954-16,210), and an average length of 111.1
189 (median: 115.5; range = 74 - 138).

190 ***Nasopharyngeal microbiota***

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191 In total, 22 phyla were detected; however, five predominant phyla were identified as the
192 most abundant in all groups: *Proteobacteria, Firmicutes, Actinobacteria*, *Bacteroidetes* and
193 *Tenericutes* (Figure 1). Together, these major phyla comprise 96%, 97%, 95% and 80% of detected
194 phyla of BRD-14d, BRD-28d, healthy-14d and healthy-28d calves, respectively. Within
195 *Proteobacteria*, *Pseudomonas* spp. was the most abundant (11%), from which *Pseudomonas*
196 *fluorescens* showed the highest abundance. This phylum was mainly constituted of *Pseudomonas*
197 spp, *Psychrobacter* spp, *Mannheimia* spp., *Actinobacillus* spp., *Enhydrobacter* spp., *Escherichia*
198 spp., *Acinetobacter* spp., *Haemophilus* spp. and *Moraxella* spp..

199 A significant difference in the phyla relative abundance between groups was observed for
200 the phylum *Bacteroidetes* ($P < 0.05$). Comparison of *Bacteriodetes* mean relative abundance
201 between health status and sampling time point revealed significant differences between healthy-28d
202 and healthy-14d samples ($P < 0.05$).

203 Analysis at the genus level revealed 20 dominating genera (Figure 2). The most important
204 genera related to respiratory diseases, such as *Mycoplasma* spp., *Mannheimia* spp., and *Pasteurella*
205 spp. were detected in all groups (Figure 2), and numeric, although statistically not significant,
206 differences were observed.

207 Relative abundances of the 20 most abundant bacteria (species level information) detected in
208 DNP samples from healthy calves and calves that developed BRD are shown in Figures 3 and 4.
209 Relative abundance of *Pseudomonas fluorescens* for each different group of calves was 0.07 ± 0.04
210 (BRD-14d), 0.002 ± 0.002 (BRD-28d), 0.01 ± 0.005 (healthy-14d) and 0.002 ± 0.001 (healthy-28d)
211 ($P < 0.05$). Despite no significant differences being detected between the main bacteria related to
212 pneumonia in calves [*Mannheimia haemolytica* ($P = 0.60$) and *Pasteurella multocida* ($P = 0.83$)]
213 and health status, these pathogens were within the 20 most abundant species, unlike *Histophilus*
214 *somni* and *Mycoplasma bovis* (Figures 3 and 4). *Mannheimia haemolytica* was the most abundant
215 bacterium and its relative abundance was numerically increased in both BRD-28d (0.06 ± 0.03) and

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216 healthy-28d calves (0.07 ± 0.02) comparing to BRD-14d and healthy-14d calves. On the other hand,
217 *Pasteurella multocida* relative abundance was low in all four groups (Figure 4). *Moraxella*
218 *catarrhalis* was also detected in all groups of calves. Relative abundance of the bacteria most
219 commonly associated to pneumonia (namely *Mannheimia haemolytica, Pasteurella multocida*,
220 *Histophilus somni*, and *Mycoplasma bovis*) are also presented in Figure 6.

221 The proportion of calves with a positive detection of these known pathogens is presented in
222 Table 1. *Mannheimia haemolytica* was detected in most of the studied calves (78.12%). On the
223 other hand, lower proportion was observed for *Mycoplasma bovis* (18.75%). *Mycoplasma bovis* was
224 detected in 16.7% of the calves in both BRD groups and in 40% of the healthy-28d calves.

225 Analysis at the strain level revealed numeric differences between healthy animals and
226 animals that developed BRD for *Mannheimia haemolytica* non-serotype A1 str. PHL213 (P =0.11)
227 (Figure 5). Relative abundance of this specific serotype (expressed as a proportion out of all
228 *Mannheimia haemolytica* serotypes) was increased in BRD-14d calves (0.44 ± 0.14) and BRD-28d
229 (0.49 ± 0.11) compared to healthy-14d (0.35 ± 0.12) and decreased compared to healthy-28d calves
230 (0.53 ± 0.09). *Mannheimia haemolytica* serotype A1 str.PHL213 relative abundances were also
231 increased in BRD-14d calves (0.23 ± 0.07) and BRD-28d (0.34 ± 0.09) compared to healthy-14d
232 (0.15 ± 0.05) and decreased compared to healthy-28d calves (0.37 ± 0.08) (Figure 5).

233

234 ***Functional profile***

235 In addition to a taxonomic profile, shotgun sequencing provides information regarding
236 samples' functional profile. The relative abundance of the 20 most abundant functional features is
237 shown in Figure 7. Relative abundance of some functional features tended to be numerically
238 increased in samples from BRD calves compared to non-BRD calves (Amino Acids and
239 derivatives: P = 0.87; Carbohydrates: P = 0.84; Cell division and cell cycle: P = 0.95; Cell wall

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240 and capsule: P = 0.99; Clustering-based subsystems: P = 0.61; Cofactors, vitamins, prosthetic
241 groups, pigments: P = 0.88; DNA metabolism: P = 0.97; Dormancy and sporulation: P = 0.17;
242 Fatty acids and isoprenoids: P = 0.94; Iron acquisition and metabolism: P = 0.90; Membrane
243 transport: P = 0.95; Metabolism and aromatic compounds: P = 0.09; Miscellaneous: P = 0.88;
244 Motility and chemotaxis: P = 0.83; Nitrogen metabolism: P = 0.93; Nucleosides and nucleotides: P
245 = 0.87; Phages, prophages, transposable elements and plasmids: P = 0.88; Phosphorus metabolism:
246 P = 0.67; Photosynthesis: P = 0.82; Potassium metabolism: P = 0.99; Protein metabolism: P =
247 0.73; RNA metabolism: P = 0.81; Regulation and cell signaling: P = 0.58; Secondary metabolism:
248 P = 0.93; Stress metabolism: P = 0.76; Sulfur metabolism: P = 0.93; Virulence and Disease and
249 Defense factors: P = 0.69).

250 Analysis of virulence and disease and defense factors revealed numeric differences in
251 resistance to antibiotics and toxic compounds features (P = 0.12) between healthy calves and calves
252 that developed BRD. Results from this analysis are presented in Figure 8. Bacteriocins was only
253 detected in BRD-14d calves. Adhesion factors were more abundant in healthy-28d calves compared
254 to the other groups (P = 0.67) (Figure 8).

255 The resistance to antibiotics and toxic compounds analysis revealed differences between
256 BRD-14d and healthy-14d calves in cobalt-zinc-cadmium resistance (Figure 9). These differences
257 tended to be statistically significant (P = 0.07). The relative abundance of multidrug resistance
258 efflux pumps was numerically increased in BRD-14d (0.11 ± 0.08) and BRD-28d (0.10 ± 0.08)
259 comparing to healthy calves (P = 0.57). Resistance to fluoroquinolones was numerically increased
260 in healthy-28d calves (0.38 ± 0.13) and BRD-28d (0.29 ± 0.16) (P = 0.57). Methicilin-resistant
261 Staphylococcus (P = 0.29) and copper homeostasis features (P = 0.66) were both numerically
262 increased in BRD-28d (0.19 ± 0.16; 0.04 ± 0.04, respectively). Beta-lactamase (P = 0.55) was only
263 detected in healthy-14d calves (0.01 ± 0.01). Cadmium resistance was numerically increased in
264 BRD-28d calves (0.08 ± 0.08) compared to the other groups (P = 0.25) (Figure 9).

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266 **DISCUSSION**

267 In an effort to better understand the upper respiratory tract metagenome in dairy calves, we
268 used shotgun sequencing of DNP samples and characterized the differences between healthy
269 animals at 14 and 28 days of life and animals that developed BRD between these two time points.
270 These two time points were selected mainly because all BRD cases in the Lima et al. (2016) study
271 were diagnosed between the 14th and the 28th day of life. Our data revealed a remarkable
272 complexity in the bacterial ecology of both healthy and BRD affected calves. *Moraxella*
273 *catarrhalis,* a species well known to be associated with upper respiratory tract disease in humans,
274 was detected in all groups, particularly in BRD calves, although this difference was not statistically
275 significant. Upon functional profile analysis, we observed greater abundance of Cobalt-Zinc-
276 Cadmium resistant genes in BRD-14d samples, which could indicate difficulties in treatment of
277 these animals, given that the presence of these genes has been associated with increased resistance
278 to antibiotics.

279 It should be noted that our study could have been more informative had we been able to
280 obtain bronchoalveolar lavage samples (at least at the second sampling time point) which would
281 have allowed us to study the lung metagenome in addition to the upper respiratory tract
282 metagenome. However, our study was conducted on a commercial dairy farm and it was not
283 possible for us to secure the owner's consent for such a sampling approach. The small number of
284 enrolled calves represents another limitation of our study. Although shotgun metagenomic studies
285 are commonly conducted on small number of subjects (as the cost of conducting such studies
286 remains high) this limitation should be acknowledged and results presented here should be
287 interpreted with caution. It should also be noted that our study was conducted on a single farm; a
288 larger scale, multi-farm study would have potentially been more informative. Sampling time points
289 were fixed (14 and 28 days of life) and dictated by the Lima et al. 2016 study design. This

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290 represents another limitation of our study since calves developed BRD and were treated with
291 antibiotics at different time points (between 14 and 28 days of life).

292 Calves that were diagnosed with BRD in our study received antibiotic therapy. Systemic
293 antibiotic therapy in calves should impact the microbiome of the URT. Therefore the post treatment
294 upper respiratory tract microbiome is likely a reflection of both the micro-organisms involved in the
295 disease process and the impact of antimicrobial treatment. We have recently shown that parenteral
296 administration of antibiotics has a temporal effect on the neonatal calf's fecal microbiome and this
297 should also be the case for the upper respiratory tract microbiome (Oultram et al. 2015). Our study
298 was conducted on a commercial dairy farm and farm protocols could not be modified solely for the
299 purpose of the study. Additionally, BRD is commonly caused by bacterial infection and neglecting
300 to treat sick calves with the proper antibiotic therapy could be considered inhumane and may not
301 have been approved by the Cornell Institutional Animal Care and Use Committee. Differences
302 between samples obtained at different time points could also be due to the evolution of the
303 nasopharyngeal microbiota. Timsit et al. (2016) recently reported that the nasopharyngeal
304 microbiota of beef cattle underwent a profound evolution from weaning until arrival at a feedlot and
305 from arrival to day 40.

306 Analysis at phylum-level detected 22 phyla, of which *Proteobacteria, Firmicutes,*
307 *Actinobacteria, Bacteroidetes* and *Tenericutes* were the most abundant. Our results are in agreement
308 with those by Holman et al. (2015a) who, studying the nasopharyngeal microbiome of healthy and
309 BRD calves, detected 22 phyla of which *Proteobacteria* and *Firmicutes* were the major ones.
310 Studies in pigs (Lowe et al., 2011) and children (Bogaert et al., 2011) revealed similar results at the
311 phylum level. Conversely, the phylum *Tenericutes* was dominating the nasopharyngeal microbiota
312 of beef cattle from weaning to 40 days after their arrival at a feedlot (Timsit et al., 2016).

313 Analysis at the genus-level revealed 20 major genera that belonged to the aforementioned
314 five phyla. Among them, significant differences were detected in *Pseudomonas* spp. relative

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315 abundance between different groups of calves. *Pseudomonas fluorescens* was the species
316 responsible for this significant difference being more abundant in BRD-14d calves. Reduction in its
317 abundance in BRD-28d calves could be associated with antibiotic treatment. *Pseudomonas*
318 *fluorescens* has been associated with the production of an antifungal lipopetide for biocontrol of
319 plant pathogenic fungus (Nielsen et al., 2000). In humans, urinary tract infections (Von Graevenitz
320 and Weinstein, 1971), bacteremia in oncology patients (Hsueh et al., 1998) and a possible role in
321 the intestinal mucosal damage in Crohn's disease patients (Wei et al., 2002; Scales et al., 2014)
322 have all been attributed to *Pseudomonas fluorescens.* Although *Pseudomonas fluorescens* has been
323 identified by culture-independent and culture-dependent techniques in broncheoalveolar lavage
324 fluid (Bahrani-Mougeot et al., 2007) and tracheal aspirates (Redding and McWalter, 1980) in
325 patients with pneumonia, its role in respiratory disease remains unclear. In ruminants, *Pseudomonas*
326 *fluorescens* has not been reported as a causative agent of any infections.

327 *Mannheimia haemolytica, Mycoplasma bovis, Histophilus somni* and *Pasteurella multocida*
328 are the most commonly reported bacteria in pneumonia cases in cattle (Rice et al., 2007; Griffin et
329 al., 2010). In the present study *Mannheimia haemolytica* was the most abundant bacterium in all
330 groups and was detected in most of the studied calves. Its relative abundance was increased in both
331 group of calves, particularly in the healthy-28d one. *Mannheimia haemolytica* is an upper
332 respiratory tract commensal that can become pathogenic under certain circumstances (Griffin et al.,
333 2010). Several studies have reported the presence of *Mannheimia haemolytica* in the respiratory
334 tract of both healthy and BRD calves, most of which observed an association between this
335 bacterium and pneumonia (Angen et al., 2009; Francoz et al., 2015; Holman et al., 2015a). The
336 analysis of *Mannheimia haemolytica* strains showed the presence of serotype A2 in all groups. This
337 is the predominant strain detected in healthy calves (Rice et al., 2007) and it is associated with
338 pneumonia in sheep (Lawrence et al., 2010). On the other hand, pneumonia in cattle is highly
339 associated with serotypes A1 and A6 (Griffin, 2010). Interestingly, we detected numeric differences

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340 in *Mannheimia haemolytica* serotype A1 str. PHL 213 relative abundance between healthy and
341 BRD calves, which is in accordance with previous research (Purdy et al., 1997; Al-Ghamdi et al.,
342 2000; Griffin et al., 2010).

343 Despite being commonly reported as a causative agent of pneumonia in calves (Kusiluka et
344 al., 2000; Gagea et al., 2006), we could only detect *Mycoplasma bovis* in very low relative
345 abundances. *Mycoplasma bovis* sequences were detected in one out of six BRD-14d calves, one out
346 of six BRD-28d calves, and four out of ten healthy 28d calves. *Mycoplasma bovis* sequences were
347 absent from all the healthy-14d calves. The rather low relative abundance of *Mycoplasma bovis*
348 observed and the presence of other pneumonia-associated relevant bacteria, support the hypothesis
349 that *Mycoplasma bovis*, is an opportunistic pathogen (Rosendal and Martin, 1986) and part of the
350 normal upper respiratory tract microbiome of calves.

351 *Histophilus somni* is a commensal microorganism that belongs to the nasopharyngeal
352 microbiome of calves (Griffin et al., 2010) and like other bacteria that reside in the upper
353 respiratory tract, becomes pathogenic after changes in the upper respiratory tract environment. In
354 the present study, *Histophilus somni* was less abundant than *Mannheimia haemolytica*, *Mycoplasma*
355 *bovis*, and *Pasteurella multocida*.

356 *Pasteurella multocida* was one of the 20 most abundant bacteria detected in the deep nasal
357 swabs samples. It was detected in both healthy and sick groups, although it was not identified in all
358 samples. *Pasteurella multocida* was detected in BRD calves in slightly higher abundance;
359 particularly on 14 days of life; however no statistically significant differences between groups were
360 detected. Several studies detected *Pasteurella multocida* in the respiratory tract of calves, using
361 culture-dependent and culture-independent techniques; in most of them, this bacterium was highly
362 prevalent (Angen et al., 2009; Jamali et al., 2014; Holman et al., 2015b).

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363 *Moraxella catarrhalis* is a well-known Gram-negative pathogen associated with respiratory
364 disease in humans (Verduin et al., 2002; Goldstein et al., 2009), particularly in children (Sy and
365 Robinson, 2010). Although it is described as an exclusive pathogen of the upper respiratory tract of
366 humans (Goldstein et al., 2009), *Moraxella catarrhalis* was isolated from lungs of BRD calves and
367 its presence was also demonstrated in lung lesions from the same calves by immunohistology in the
368 study conducted by Nagai et al., (1995). Recently, *Moraxella* spp. was detected in the
369 nasopharyngeal tract of calves in a feedlot (Holman et al., 2015a), however, the species was not
370 determined and the health status of the calves was not reported.

371 *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae* and *Haemophilus parasuis*
372 were among the 20 most abundant bacteria detected in DNP swab samples. These species are well-
373 known pathogens associated with swine respiratory disease (Bossé et al., 2002; Nedbalcova et al.,
374 2006; Sibila et al., 2009). Although *Actinobacillus pleuropneumoniae* is considered an exclusive
375 pathogen of the swine respiratory tract, this species has previously been isolated from cattle, deer
376 and lambs (Desrosiers et al., 1998). The potential role of these fastidious bacteria in BRD may
377 warrant further investigation.

378 Functional profiling showed numeric differences between healthy calves and calves that
379 developed BRD, the latter having increased abundances of resistance to antibiotics and toxic
380 compounds features. Cadmium, zinc and cobalt resistance is observed in bacteria such as
381 *Staphylococcus aureus* and *Escherichia coli* and is related to proteins such as *cadCA cadB, czcCBA*
382 and *cnr*, depending on the bacteria (Nies, 1992). Five mechanisms of metal-resistance are described
383 (Choudhury and Srivastavara, 2001): exclusion of metal by permeable barrier, exclusion by active
384 transport, intra and extracellular physical sequestration and transformation and detoxification.
385 Studies reported heavy metals as a source of co-selection of antibiotic resistance (Pettibone et al.,
386 1996; Stepanauskas et al., 2005; Seiler and Berendonk, 2012). Co-selection occurs due to
387 physiological (cross-resistance) and genetic (co-resistance) mechanisms. The first provides

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388 resistance to more than one antibiotic agent (drugs and heavy metal) using mechanisms such as
389 Multi Drug Efflux Pumps (Baker-Austin et al., 2006; Martinez et al., 2009) and the second one is
390 related to two or more genetically linked resistance genes (Chapman, 2003). In the present study,
391 genes related to heavy metal resistance were increased in BRD calves compared with healthy calves
392 and this could imply difficulties in the treatment of diseased calves, given that studies have
393 highlighted the importance of co-selection and heavy metal resistance for antibiotic resistance
394 (Chapman, 2003). Genes associated with antibiotic resistance were also more abundant in samples
395 obtained from BRD calves.

396

397 **CONCLUSION**

398 In this particular study, *Mannheimia haemolytica* and *Pasteurella multocida* were part of the
399 20 most abundant bacteria found in healthy and BRD calves. *Moraxella catarrhalis*, an important
400 bacterium for human pneumonia, was detected in both groups, particularly in BRD calves. The
401 importance of *Moraxella catarrhalis* for calves' respiratory disease remains elusive. Cobalt-Zinc-
402 Cadmium resistant genes observed mostly in BRD calves might be associated with difficulties in
403 the treatment using antibiotics.

404 **ACKNOWLEDGEMENTS**

405 The São Paulo Research Foundation (FAPESP) is gratefully acknowledged for providing
406 funding (Protocol # 2015/02947-0).

407

408 **REFERENCES**

409 Aebi, M., B.H. van den Borne, A. Raemy, A. Steiner, P. Pilo, and M. Bodmer. 2015. *Mycoplasma*
410 *bovis* infections in Swiss dairy cattle: a clinical investigation. Acta Vet. Scand. 57:1–11.
411 doi:10.1186/s13028-015-0099-x.

For Peer Review

412 Al-Ghamdi, G.M., T.R. Ames, J.C. Baker, R. Walker, C.C. Chase, G.H. Frank, and S.K.
413 Maheswaran. 2000. Serotyping of *Mannheimia (Pasteurella) haemolytica* isolates from the
414 upper Midwest United States. J. Vet. diagnostic Investig. 12:576–8.
415 doi:10.1177/104063870001200617.

416 Angen, O., J. Thomsen, L.E. Larsen, J. Larsen, B. Kokotovic, P.M.H. Heegaard, and J.M.D.
417 Enemark. 2009. Respiratory disease in calves: microbiological investigations on trans-
418 tracheally aspirated bronchoalveolar fluid and acute phase protein response. Vet. Microbiol.
419 137:165–71. doi:10.1016/j.vetmic.2008.12.024.

420 Bahrani-Mougeot, F.K., B.J. Paster, S. Coleman, S. Barbuto, M.T. Brennan, J. Noll, T. Kennedy,
421 P.C. Fox, and P.B. Lockhart. 2007. Molecular Analysis of Oral and Respiratory Bacterial
422 Species Associated with Ventilator-Associated Pneumonia. J. Clin. Microbiol. 45:1588–1593.
423 doi:10.1128/JCM.01963-06.

424 Baker-Austin, C., M.S. Wright, R. Stepanauskas, and J. V McArthur. 2006. Co-selection of
425 antibiotic and metal resistance. Trends Microbiol. 14:176–82. doi:10.1016/j.tim.2006.02.006.

426 Bogaert, D., B. Keijser, S. Huse, J. Rossen, R. Veenhoven, E. van Gils, J. Bruin, R. Montijn, M.
427 Bonten, and E. Sanders. 2011. Variability and diversity of nasopharyngeal microbiota in
428 children: a metagenomic analysis. PLoS One. 6:e17035. doi:10.1371/journal.pone.0017035.

429 Bosch, A.A.T.M., G. Biesbroek, K. Trzcinski, E.A.M. Sanders, and D. Bogaert. 2013. Viral and
430 Bacterial Interactions in the Upper Respiratory Tract. PLoS Pathog. 9.
431 doi:10.1371/journal.ppat.1003057.

432 Bossé, J.T., Janson, H., Sheeran, B.J., Beddek, A.J., Rycroft, A.N., Kroll, S., Langford. 2002.
433 *Actinobacillus pleuropneumoniae*: pathobiology and pathogenesis of infection. doi:
434 10.1016/S1286-4579(01)01534-9

435 Cernicchiaro, N., B.J. White, D.G. Renter, and A.H. Babcock. 2013. Evaluation of economic and
436 performance outcomes associated with the number of treatments after an initial diagnosis of
437 bovine respiratory disease in commercial feeder cattle. Am. J. Vet. Res. 74:300–309.
438 doi:10.2460/ajvr.74.2.300.

439 Chapman, J.S. 2003. Disinfectant resistance mechanisms, cross-resistance, and co-resistance. Int.
440 Biodeterior. Biodegradation. 51:271–276. doi:10.1016/S0964-8305(03)00044-1.

441 Choudhury, R., and S. Srivastava. 2001. Zinc resistance mechanisms in bacteria. Curr Sci. 81:768-
442 775.

443 Desrosiers, R., Moore, C. 1998. Indirect transmission of *Actinobacillus pleuropneumoniae*.
444 Swine Health and Prod. 6(6):263–265.

445 Francoz, D., S. Buczinski, A.M. Belanger, G. Forte, O. Labrecque, D. Tremblay, V. Wellemans,
446 and J. Dubuc. 2015. Respiratory pathogens in Quebec dairy calves and their relationship with
447 clinical status, lung consolidation, and average daily gain. J. Vet. Intern. Med. 29:381–387.
448 doi:10.1111/jvim.12531.

For Peer Review

ScholarOne support: (434) 964 4100

449 Gagea, M.I., K.G. Bateman, R. a Shanahan, T. van Dreumel, B.J. McEwen, S. Carman, M.
450 Archambault, and J.L. Caswell. 2006. Naturally occurring *Mycoplasma bovis*-associated
451 pneumonia and polyarthritis in feedlot beef calves. J. Vet. Diagn. Invest. 18:29–40.
452 doi:10.1177/104063870601800105.

453 Gershwin, L.J., A.L. Van Eenennaam, M.L. Anderson, H.A. McEligot, M.X. Shao, R. Toaff-
454 Rosenstein, J.F. Taylor, H.L. Neibergs, and J. Womack. 2015. Single Pathogen Challenge with
455 Agents of the Bovine Respiratory Disease Complex. PLoS One. 10:e0142479.
456 doi:10.1371/journal.pone.0142479.

457 Goldstein, E.J.C., T.F. Murphy, and G.I. Parameswaran. 2009. *Moraxella catarrhalis*, a Human
458 Respiratory Tract Pathogen. Clin. Infect. Dis. 49:124–131. doi:10.1086/599375.

459 Griffin, D. 1997. Economic impact associated with respiratory disease in beef cattle. Vet. Clin.
460 North Am. Food Anim. Pract. 13:367–77.

461 Griffin, D. 2010. Bovine Pasteurellosis and Other Bacterial Infections of the Respiratory Tract. Vet.
462 Clin. North Am. - Food Anim. Pract. 26:57–71. doi:10.1016/j.cvfa.2009.10.010.

463 Griffin, D., M.M. Chengappa, J. Kuszak, and D.S. McVey. 2010. Bacterial pathogens of the bovine
464 respiratory disease complex. Vet. Clin. North Am. Food Anim. Pract. 26:381–94.
465 doi:10.1016/j.cvfa.2010.04.004.

466 Holman, D.B., T. McAllister, E. Topp, A.-D.G. Wright, and T.W. Alexander. 2015a. The
467 nasopharyngeal microbiota of feedlot cattle that develop bovine respiratory disease. Vet.
468 Microbiol. doi:10.1016/j.vetmic.2015.07.031.

469 Holman, D.B., E. Timsit, and T.W. Alexander. 2015b. The nasopharyngeal microbiota of feedlot
470 cattle. Sci. Rep. doi:10.1038/srep15557.

471 Hsueh, P-R., Teng, L-J, Pan H-J., Chen, Y.-C., Sun, C.-C., Ho, S.-W., and Luh, K.-T. 1998.
472 Outbreak of *Pseudomonas fluorescens* Bacteremia among Oncology Patients. J. Clin. Microb.
473 36 :2914-2917.

474 Jamali, H., M. Rezagholipour, S. Fallah, and A. Dadrasnia. 2014. Prevalence , characterization and
475 antibiotic resistance of *Pasteurella multocida* isolated from bovine respiratory infection. Vet. J.
476 202:381–383. doi:10.1016/j.tvjl.2014.07.024.

477 Kusiluka, L.J., B. Ojeniyi, and N.F. Friis. 2000. Increasing prevalence of Mycoplasma bovis in
478 Danish cattle. Acta Vet. Scand. 41:139–46.

479 Lawrence, P.K., W. Kittichotirat, R.E. Bumgarner, J.E. McDermott, D.R. Herndon, D.P. Knowles,
480 and S. Srikumaran. 2010. Genome sequences of *Mannheimia haemolytica* serotype A2: Ovine
481 and bovine isolates. J. Bacteriol. 192:1167–1168. doi:10.1128/JB.01527-09.

482 Lima, S.F., A.G. V. Teixeira, C.H. Higgins, F.S. Lima, and R.C. Bicalho. 2016. The upper
483 respiratory tract microbiome and its potential role in bovine respiratory disease and otitis
484 media. Sci. Rep. 6:29050. doi:10.1038/srep29050.

485 Lowe, B.A., T.L. Marsh, N. Isaacs-Cosgrove, R.N. Kirkwood, M. Kiupel, and M.H. Mulks. 2011.

For Peer Review

486 Microbial communities in the tonsils of healthy pigs. Vet. Microbiol. 147:346–357.
487 doi:10.1016/j.vetmic.2010.06.025.

488 Martinez, J.L., M.B. Sánchez, L. Martínez-Solano, A. Hernandez, L. Garmendia, A. Fajardo, and C.
489 Alvarez-Ortega. 2009. Functional role of bacterial multidrug efflux pumps in microbial natural
490 ecosystems. FEMS Microbiol. Rev. 33:430–49. doi:10.1111/j.1574-6976.2008.00157.x.

491 Nagai, M., T. Ozawa, N. Ishikawa, H. Komae, Y. Hayakawa, and M. Nakazuwa. 1995. Isolation of
492 *Moraxella catarrhalis* from Calves with Pneumonia. J. Japan Vet. Med. Assoc. 48:319–322.
493 doi:10.12935/jvma1951.48.319.

494 Nedbalcova, K., Satran, P., Jaglic, Z., Ondriasova, R., Kucerova, Z. 2006. Haemophilus parasuis
495 and Glässer's disease in pigs: a review. Vet. Med. 51(5):168–179

496 Nielsen, T.H., C. Thrane, C. Christophersen, U. Anthoni, and J. Sorensen. 2000. Structure,
497 production characteristics and fungal antagonism of tensin - a new antifungal cyclic
498 lipopeptide from *Pseudomonas fluorescens* strain 96.578. J. Appl. Microbiol. 89:992–1001.
499 doi:10.1046/j.1365-2672.2000.01201.x.

500 Nies, D.H. 1992. Resistance to cadmium, cobalt, zinc, and nickel in microbes. Plasmid. 27:17–28.

501 Oultram, J., E. Phipps, A. G. V. Teixeira, C. Foditsch, M. L. Bicalho, V. S. Machado, R. C. Bicalho
502 and G. Oikonomou. 2015. Effects of antibiotics (oxytetracycline, florfenicol or tulathromycin)
503 on neonatal calves' faecal microbial diversity. The Vet. Rec. doi:10.1136/vr.103320

504 Pettibone, G.W., J.P. Mear, and B.M. Sampsell. 1996. Incidence of antibiotic and metal resistance
505 and plasmid carriage in *Aeromonas* isolated from brown bullhead (*Ictalurus nebulosus*). Lett.
506 Appl. Microbiol. 23:234–240. doi:10.1111/j.1472-765X.1996.tb00073.x.

507 Purdy, C.W., R.H. Raleigh, J.K. Collins, J.L. Watts, and D.C. Straus. 1997. Serotyping and enzyme
508 characterization of *Pasteurella haemolytica* and *Pasteurella multocida* isolates recovered from
509 BRD lungs of stressed feeder calves. Curr. Microbiol. 34:244–249.
510 doi:10.1007/s002849900177.

511 Redding, P.J., and P.W. McWalter. 1980. *Pseudomonas fluorescens* cross-infection due to
512 contaminated humidifier water. BMJ. 281:275–275. doi:10.1136/bmj.281.6235.275.

513 Rice, J.A., L. Carrasco-Medina, D.C. Hodgins, and P.E. Shewen. 2007. *Mannheimia haemolytica*
514 and bovine respiratory disease. Anim. Health Res. Rev. 8:117–128.
515 doi:10.1017/S1466252307001375.

516 Rosendal, S. and S. Martin. 1986. The association between serological evidence of mycoplasma
517 infection and respiratory disease in feedlot calves. Can. J. Vet. Res. 50:179–183.

518 Scales, B.S., R.P. Dickson, J.J. LiPuma, and G.B. Huffnagle. 2014. Microbiology, Genomics, and
519 Clinical Significance of the *Pseudomonas fluorescens* Species Complex, an Unappreciated
520 Colonizer of Humans. Clin. Microbiol. Rev. 27:927–948. doi:10.1128/CMR.00044-14.

521 Seiler, C., and T.U. Berendonk. 2012. Heavy metal driven co-selection of antibiotic resistance in
522 soil and water bodies impacted by agriculture and aquaculture. Front. Microbiol. 3.

For Peer Review

523 doi:10.3389/fmicb.2012.00399.

524 Sharpton, T.J. 2014. An introduction to the analysis of shotgun metagenomic data. Front. Plant Sci.
525 5. doi:10.3389/fpls.2014.00209.

526 Sibila, M., Pieters, M., Molitor, T., Maes, D., Haesebrouck, F., Segalés, F. 2008. Current
527 perspectives on the diagnosis and epidemiology of *Mycoplasma hyopneumoniae* infection. doi:
528 10.1016/j.tvjl.2008.02.020

529 Stepanauskas, R., T.C. Glenn, C.H. Jagoe, R.C. Tuckfield, A.H. Lindell, and J. V. McArthur. 2005.
530 Elevated microbial tolerance to metals and antibiotics in metal-contaminated industrial
531 environments. Environ. Sci. Technol. 39:3671–3678. doi:10.1021/es048468f.

532 Sy, M.G., and J.L. Robinson. 2010. Community-acquired *Moraxella catarrhalis* pneumonia in
533 previously healthy children. Pediatr. Pulmonol. 45:674–8. doi:10.1002/ppul.21243.

534 Timsit, E., D. B. Holman, J. Hallewell, and T. W. Alexander. 2016. The nasopharyngeal microbiota
535 in feedlot cattle and its role in respiratory health. Animal Frontiers 6:44-50.
536 doi:10.2527/af.2016-0022.

537 USDA. 2013. Feedlot 2001. Part IV: Health and Health Management on U.S. Feedlots with a
538 capacity of 1,000 or more head. In APHIS. National Animal Health Monitoring System, Fort
539 Collins.

540 Verduin, C.M., C. Hol, A. Fleer, H. van Dijk, and A. van Belkum. 2002. *Moraxella catarrhalis*:
541 from Emerging to Established Pathogen. Clin. Microbiol. Rev. 15:125–144.
542 doi:10.1128/CMR.15.1.125-144.2002.

543 Virtala, A., G.D. Mechor, Y.T. Gröhn, and H.N. Erb. 1996a. Morbidity from nonrespiratory
544 diseases and mortality in dairy heifers during the first three months of life. J. Am. Vet. Med.
545 Assoc. 208:2043–2046.

546 Virtala, A. M., G.D. Mechor, Y.T. Gröhn, and H.N. Erb. 1996b. The effect of calfhood diseases on
547 growth of female dairy calves during the first 3 months of life in New York State. J. Dairy Sci.
548 79: 1040-1049.

549 Von Graevenitz A. and Weinstein, J. 1971. Pathogenic significance of *Pseudomonas fluorescens*
550 and *Pseudomonas putida*. *The Yale J. Biol. Med*. 44(3):265-273.

551 Waltner-Toews, D., S.W. Martin, ,and A.H. Meek. 1986. The effect of early calfhood health status
552 on survivorship and age at first calving. Can. J. Vet. Res. 50: 314-317.

553 Wei, B., T. Huang, H. Dalwadi, C.L. Sutton, D. Bruckner, and J. Braun. 2002. Pseudomonas
554 fluorescens Encodes the Crohn's Disease-Associated I2 Sequence and T-Cell Superantigen.
555 *Infect. Immun.* 70:6567–6575. doi:10.1128/IAI.70.12.6567-6575.2002.

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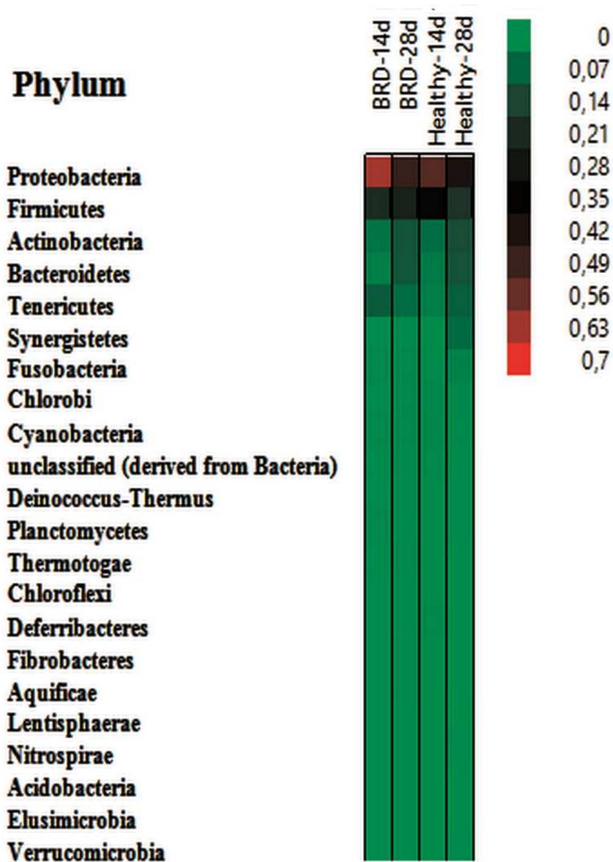
558 **Figure 1.** Heat map demonstrating the relative abundances of different phyla in deep
559 nasopharyngeal swab samples obtained from: healthy calves at 14 days of life (healthy-14d); calves
560 that developed respiratory disease between 14 and 26 days of life (samples obtained at 14 days of
561 life, BRD-14d); healthy calves at 28 days of life (healthy-28d); and calves that developed BRD
562 between 14 and 26 days of life (samples obtained at 28 days of life, BRD-28d).



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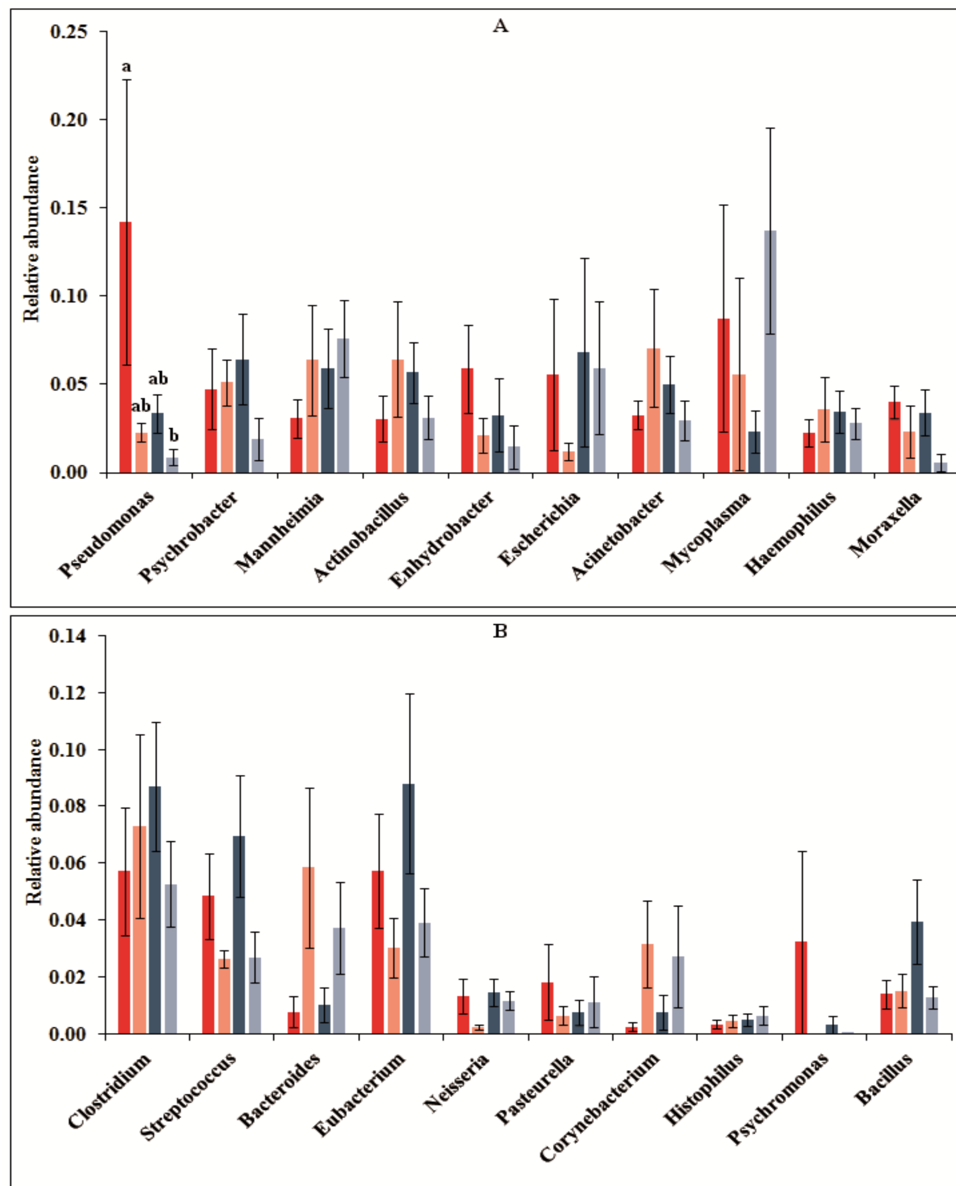
565 **Figure 2.** Relative abundance of the 20 most abundant genera (the ten most abundant of them are
566 presented in subfigure A with the rest presented in subfigure B) in deep nasopharyngeal swab
567 samples obtained from healthy calves (blue) and calves that developed respiratory disease (red) at
568 day 14 (dark colors) and day 28 (light colors) of life. Error bars represent standard error of the
569 mean. Different letters indicate Tukey-adjusted significant difference between groups ($P < 0.05$).



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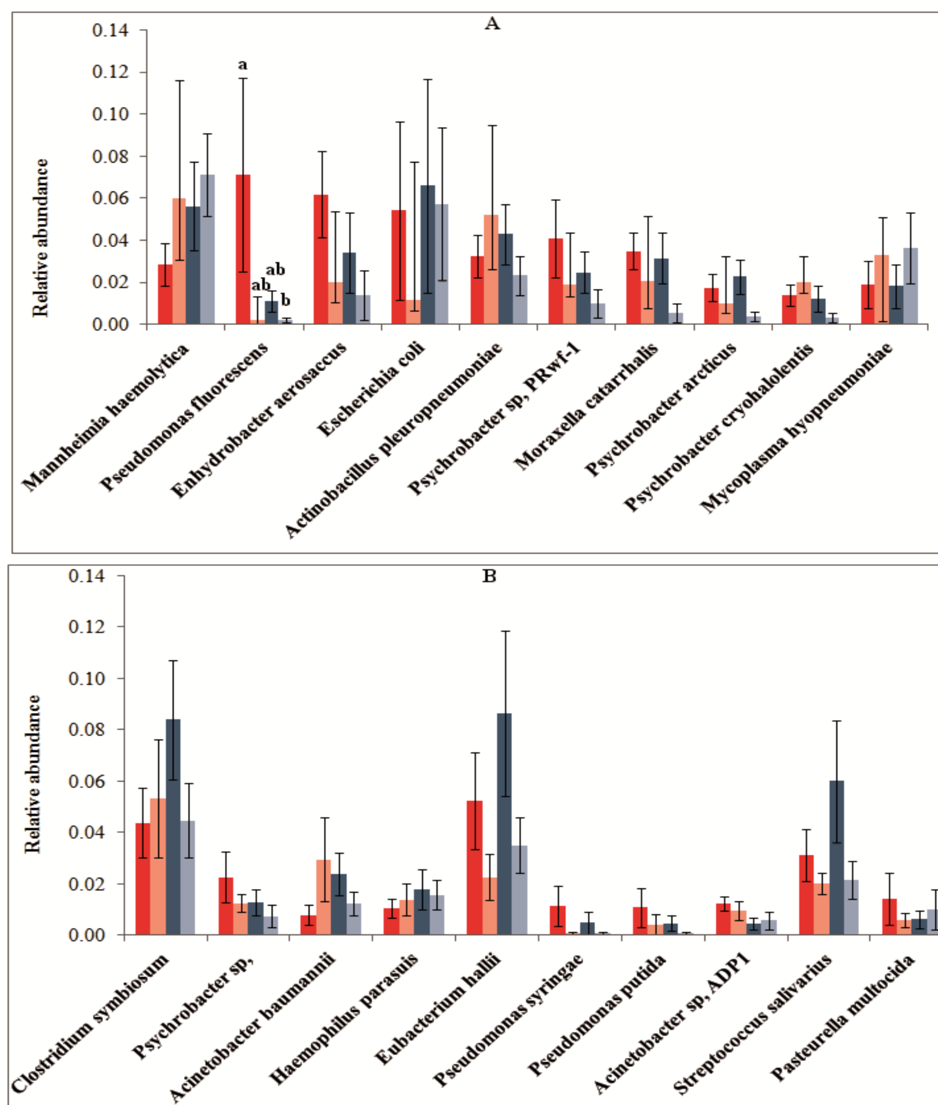
572 **Figure 3.** Relative abundance of the 20 most abundant species (the ten most abundant of them are
573 presented in subfigure A with the rest presented in subfigure B) in deep nasopharyngeal swab
574 samples obtained from healthy calves (blue) and calves that developed respiratory disease (red) at
575 day 14 (dark colors) and day 28 (light colors) of life. Error bars represent standard error of the
576 mean. Different letters indicate Tukey-adjusted significant difference between groups ($P < 0.05$).



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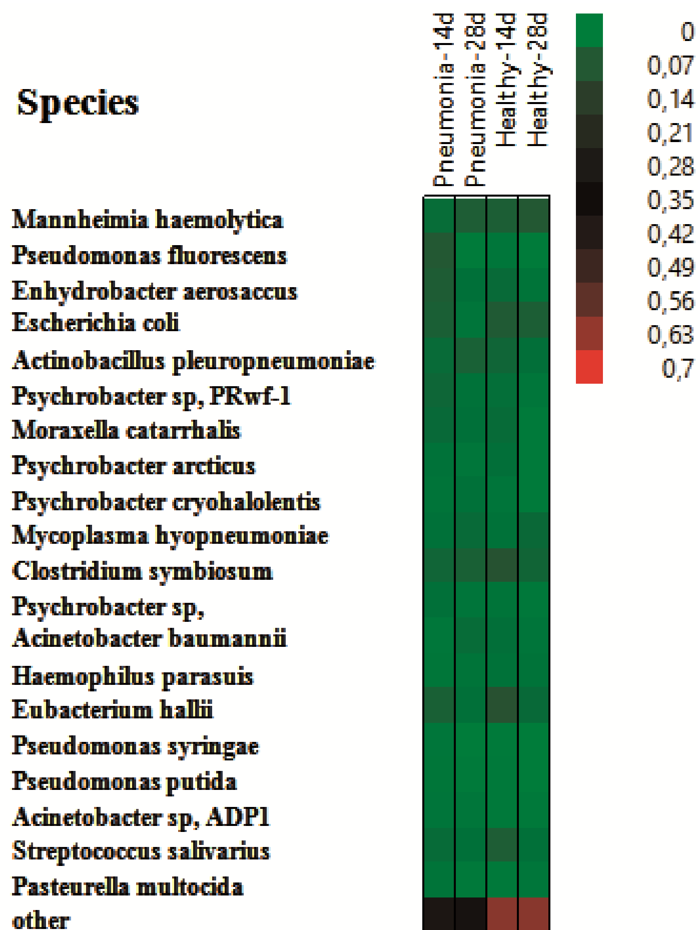
579 **Figure 4.** Heat map demonstrating the relative abundances of different species in deep
580 nasopharyngeal swab samples obtained from: healthy calves at 14 days of life (healthy-14d); calves
581 that developed respiratory disease between 14 and 26 days of life (samples obtained at 14 days of
582 life, BRD-14d); healthy calves at 28 days of life (healthy-28d); and calves that developed BRD
583 between 14 and 26 days of life (samples obtained at 28 days of life, BRD-28d).



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587 **Figure 5.** Relative abundance of *Mannheimia haemolytica* non-A1 and *Mannheimia haemolytica*
588 A1 in deep nasopharyngeal swab samples obtained from healthy calves (blue) and calves that
589 developed respiratory disease (red) at day 14 (dark colors) and day 28 (light colors) of life. Error
590 bars represent standard error of the mean. Statistically significant differences were not observed.

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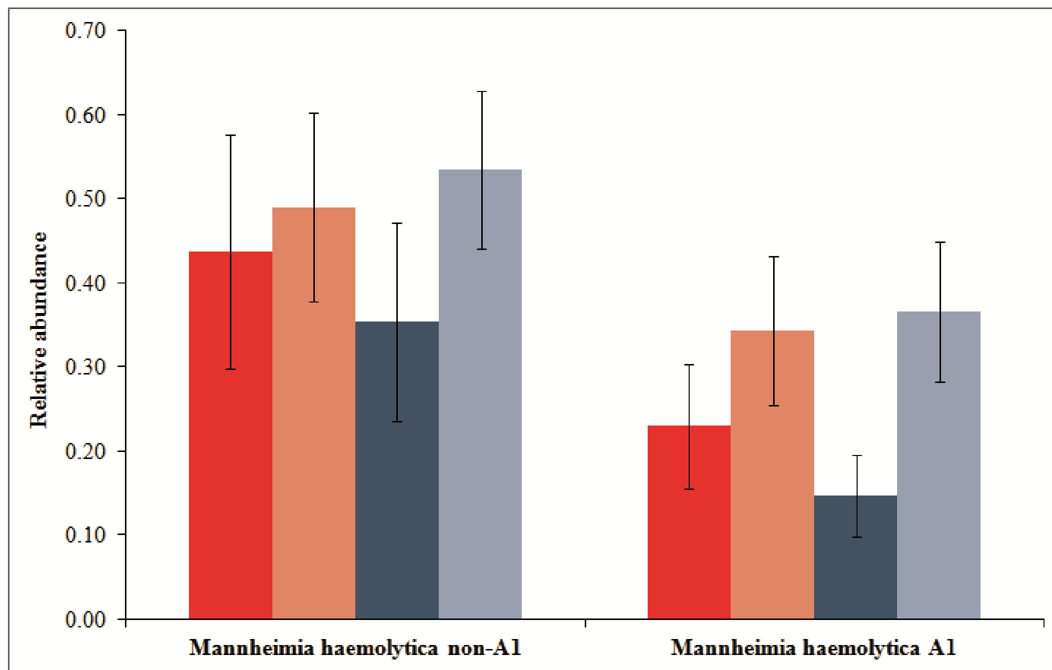

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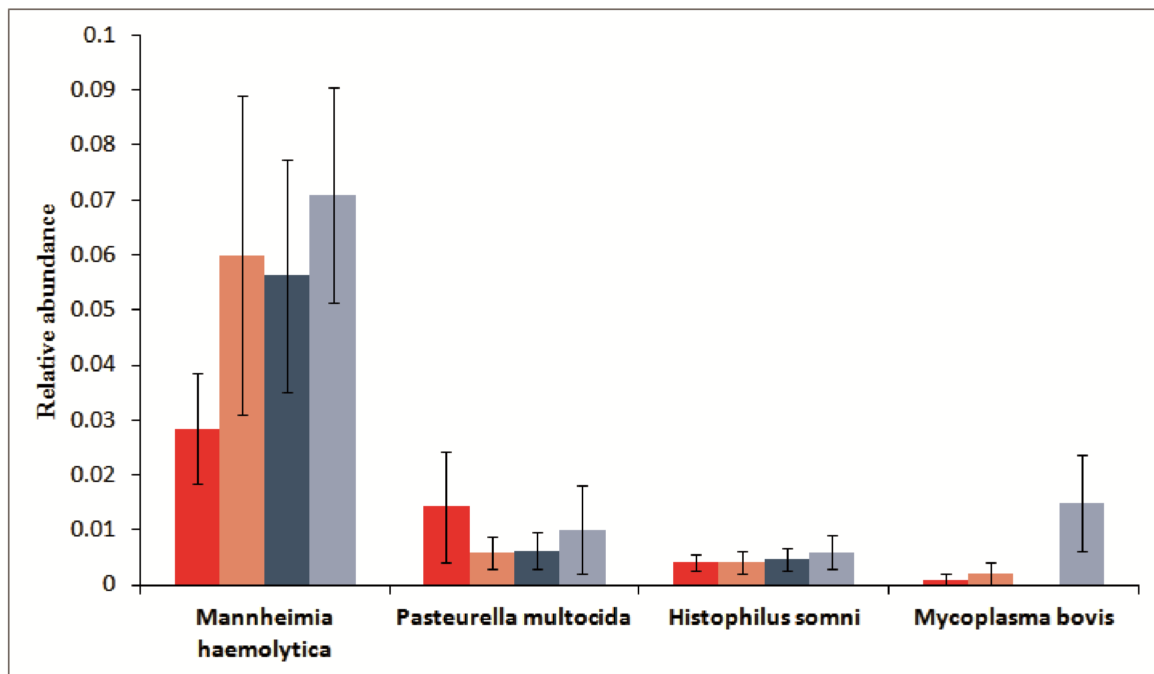
596 **Figure 6.** Relative abundance of the most common bacteria related to BRD in deep nasopharyngeal
597 swab samples obtained from healthy calves (blue) and calves that developed respiratory disease
598 (red) at day 14 (dark colors) and day 28 (light colors) of life. Error bars represent standard error of
599 the mean. Statistically significant differences were not observed.



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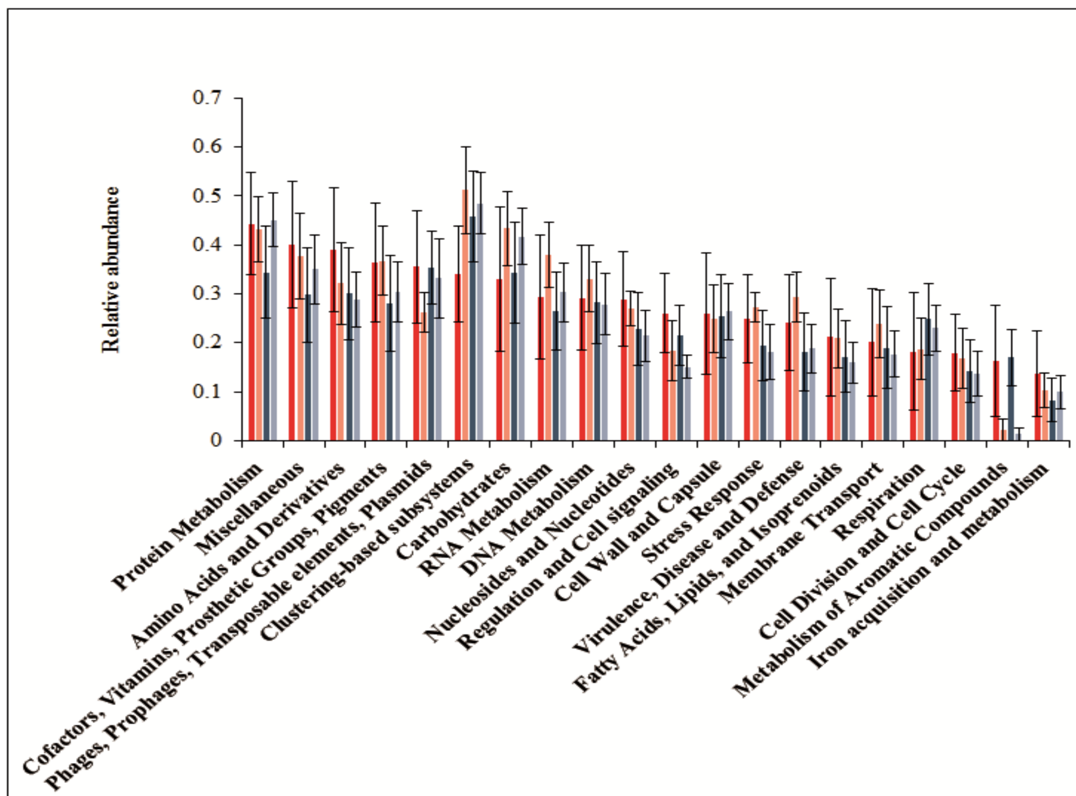
603 **Figure 7.** Relative abundance of the 20 most abundant functional profile features in deep
604 nasopharyngeal swab samples obtained from healthy (blue) and BRD calves (red) on day 14 (dark
605 colors) and day 28 (light colors) of their life. Error bars represent standard error of the mean.
606 Statistically significant differences were not observed.



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610 **Figure 8.** Normalized abundance distribution of virulence and disease and defense factors in deep
611 nasopharyngeal swab samples obtained from healthy (blue) and BRD (red) calves at day 14 (dark
612 colors) and day 28 (light colors) of their life. Error bars represent standard error of the mean.
613 Statistically significant differences were not observed.

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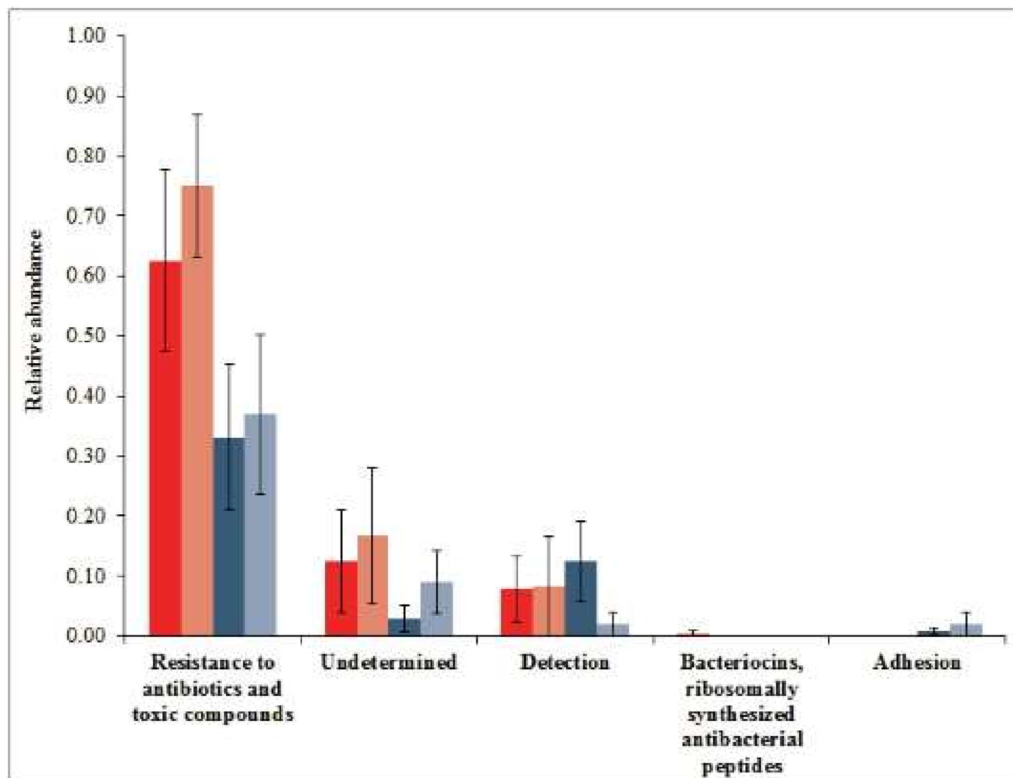

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623 **Figure 9.** Normalized abundance distribution of resistance to antibiotics and toxic compounds
624 features in deep nasopharyngeal swab samples obtained from healthy (blue) and BRD (red) calves
625 at day 14 (dark colors) and day 28 (light colors) of their life. Error bars represent standard error of
626 the mean. Statistically significant differences were not observed.

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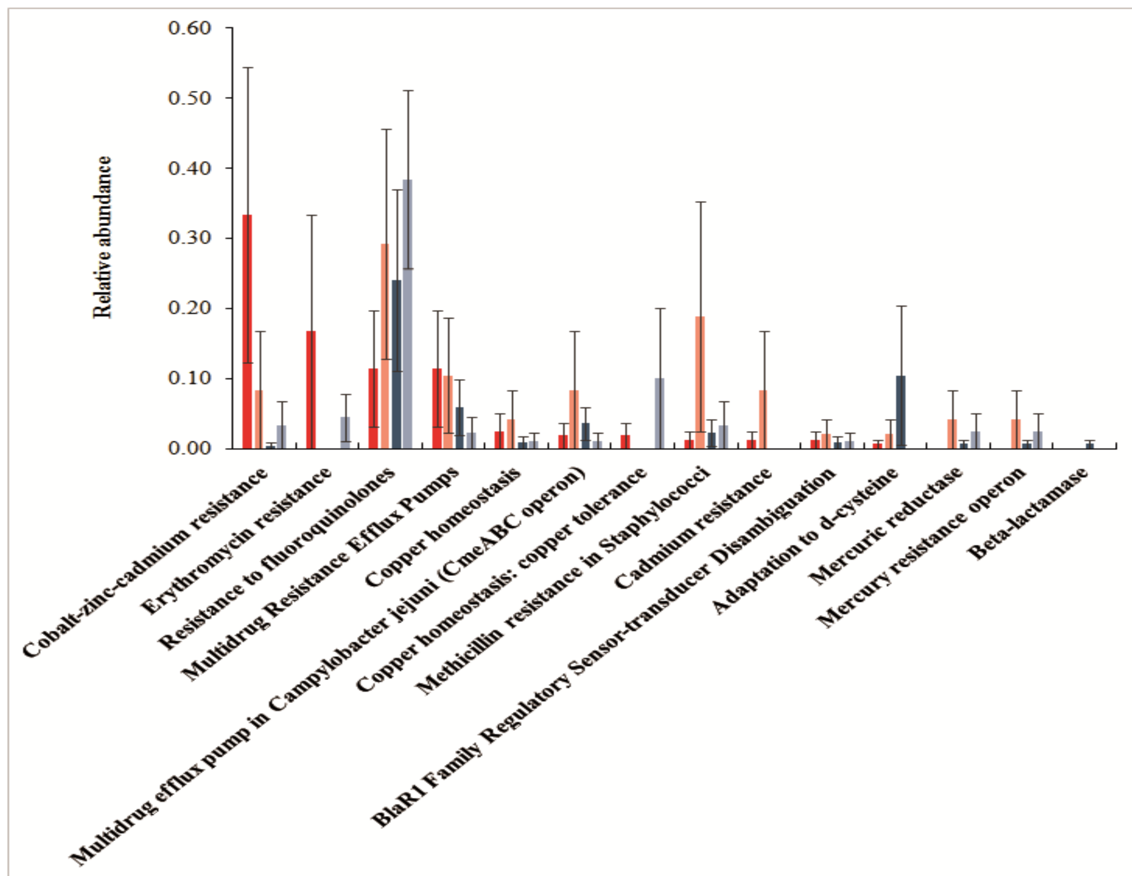

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635 **Table 1.** Proportion of calves with a positive detection of BRD bacterial pathogens according to

636 their health status (healthy and BRD calves) and sampling time (14th and 28 th day).

Pneumonic pathogens	BRD-14d N (%)	BRD-28 N (%)	Healthy-14d N (%)	Healthy-28d N (%)	Total N (%)
Mannheimia haemolytica	4 (66.7)	5 (83.3)	7 (70.0)	9 (90.0)	25 (78.12)
Histophilus somni	4 (66.7)	4 (66.7)	4 (40.0)	4 (40.0)	16 (50.0)
Pasteurella multocida	4 (66.7)	3 (50.0)	4 (40.0)	4 (40.0)	15 (46.69)
Mycoplasma bovis	1 (16.7)	1 (16.7)	0 (00.0)	4 (40.0)	6 (18.75)

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