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RESEARCH ARTICLE

Microbial associations and spatial proximity predict North American moose (*Alces alces*) gastrointestinal community composition

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Abstract

1. Microbial communities are increasingly recognized as crucial for animal health. However, our understanding of how microbial communities are structured across wildlife populations is poor. Mechanisms such as interspecific associations are important in structuring free-living communities, but we still lack an understanding of how important interspecific associations are in structuring gut microbial communities in comparison with other factors such as host characteristics or spatial proximity of hosts.
2. Here, we ask how gut microbial communities are structured in a population of North American moose *Alces alces*. We identify key microbial interspecific associations within the moose gut and quantify how important they are relative to key host characteristics, such as body condition, for predicting microbial community composition.
3. We sampled gut microbial communities from 55 moose in a population experiencing decline due to a myriad of factors, including pathogens and malnutrition. We examined microbial community dynamics in this population utilizing novel graphical network models that can explicitly incorporate spatial information.
4. We found that interspecific associations were the most important mechanism structuring gut microbial communities in moose and detected both positive and negative associations. Models only accounting for associations between microbes had higher predictive value compared to models including moose sex, evidence of previous pathogen exposure or body condition. Adding spatial information on moose location further strengthened our model and allowed us to predict microbe occurrences with ~90% accuracy.
5. Collectively, our results suggest that microbial interspecific associations coupled with host spatial proximity are vital in shaping gut microbial communities in a large

herbivore. In this case, previous pathogen exposure and moose body condition were not as important in predicting gut microbial community composition. The approach applied here can be used to quantify interspecific associations and gain a more nuanced understanding of the spatial and host factors shaping microbial communities in non-model hosts.

KEYWORDS

biotic interactions, body condition, co-occurrence networks, Markov random fields, microbiome, pathogens, spatial analysis

1 | INTRODUCTION

The relative roles of interspecific associations versus the environment in shaping communities are intensely debated (e.g. Chase & Myers, 2011; Vellend et al., 2014). In particular, detecting negative (e.g. potential competition) or positive (e.g. potential facilitation) associations between organisms remains a fundamental challenge of community ecology (e.g. Barner, Coblentz, Hacker, & Menge, 2018; Dormann et al., 2018; Harris, 2016; Ovaskainen et al., 2017). Despite the challenges of detecting associations between co-occurring species, associations between species are thought to be essential for structuring free-living communities (e.g. de Araújo, Marcondes-Machado, & Costa, 2014). For example, associations between forest animal communities were more important in structuring communities than habitat attributes such as vegetation (Le Borgne et al., 2018). In contrast, the roles that interspecific associations play in governing the assembly of microbial systems (*microbial interspecific associations*) are less understood (Ganz et al., 2017; Herren & McMahon, 2018; Zelezniak et al., 2015), particularly for within-host microbial communities. Even when microbial interspecific associations are quantified in communities, the relative importance of microbe dispersal (Evans, Martiny, & Allison, 2017; Zhou & Ning, 2017) and host characteristics in shaping microbial communities is rarely assessed (Clark, Wells, & Lindberg, 2018b). The quantification of interspecific associations of gut microbes is rare in wildlife populations but, given the significant ecological insights derived from studies of human gut microbial communities (e.g. Faust et al., 2012), is an important knowledge gap to fill.

While our understanding of how microbial communities are structured is poor, it remains a key goal in microbial ecology, particularly given microbial community relevance to animal health (e.g. Jani & Briggs, 2014; Mshelia et al., 2018). Host characteristics such as sex, body condition and the presence or absence of pathogens are recognized as important for shaping microbial communities (Britton & Young, 2014; Cheng et al., 2015; Ganz et al., 2017; Hooper, Littman, & Macpherson, 2012; Jani & Briggs, 2014, 2018; McKenney et al., 2015; Mshelia et al., 2018; Näpflin & Schmid-Hempel, 2018). For example, horses in poor body condition have greater microbial diversity and a different suite of microbial species present compared to horses in good body condition (Mshelia et al., 2018). High loads of the pathogen *Batrachochytrium dendrobatidis* can not only increase amphibian skin microbial diversity but also alter microbial composition (Jani & Briggs, 2014, 2018). Microbial community structure is also likely to be shaped by dispersal or transmission as physical distance between hosts can produce barriers to gut microbe transmission that accelerate compositional divergence (Moeller et al., 2017). However, the relative importance of environmental and host-to-host transmission compared to other ecological processes, such as host characteristics and interspecific associations, in structuring microbial communities is poorly understood.

Understanding how microbial communities are shaped, however, is technically challenging, in part due to the complexity of untangling complex ecological processes in often species-rich but poorly characterized communities (Zhou & Ning, 2017). Graphical network models such as conditional random fields (CRFs) offer exciting opportunities to address this challenge by estimating associations between organisms in a community (Clark et al., 2018b). In effect, these models can untangle relative influences of microbial interspecific associations and host characteristics in predicting community compositions. Crucially, inferences gleaned from CRFs can be integrated with phylogenetic or functional data to provide new insights into mechanisms underlying microbial community assembly. For example, associations between microbes may be non-random in that phylogenetically or functionally similar species may co-occur more frequently (e.g. Bauer & Thiele, 2018). However, non-random associations detected in the human gut have been found to be often negative and between phylogenetically or functionally similar bacterial species, indicating that competition is likely an important driver of structure in this community (Faust et al., 2012). Overlaying functional or phylogenetic information onto graphical models has the potential to highlight the broader mechanisms shaping microbial communities.

Here, we use a CRF approach to investigate microbial community composition in a wild moose (*Alces alces*) population in Minnesota. Over the last two decades, moose in Minnesota, which exist on the southern edge of their species range, have experienced significant population decreases (Delgiudice, 2018; Lenarz, 2009). The most dramatic decline has been reported for moose in north-west Minnesota, where the population declined from 4,000 animals in the 1980s to less than 100 by the mid-2000s due to a combination of pathogens and malnutrition (Murray et al., 2006). Recently, moose in

north-east Minnesota have experienced a 55% population decline, driven largely by parasitic infections in adults and wolf predation in calves (Carstensen et al., 2018; Severud, 2017; Wünschmann et al., 2015). Moose gut microbial communities are known to vary with sex and age (Ishaq, Sundset, Crouse, & Wright, 2015; Ishaq & Wright, 2012), but to what extent these host characteristics, as well as pathogens and malnutrition, shape moose gut microbial communities is unknown. Other ecological processes such interspecific associations, even though rarely quantified in wild populations, are also likely to play a role in shaping the moose gut microbial community as they do for other ruminants (Henderson et al., 2015). Furthermore, animals within close proximity to each other may have similar gut microbial communities due to similarities in diet and/or increased possibilities for microbial transmission between individuals; both factors are known to be important in gut microbial community assembly in many host species (Henderson et al., 2015; Moeller et al., 2017).

Here, we apply a novel CRF approach that can quantify the relative importance of host characteristics (including moose sex, body condition, pathogen exposure and pregnancy status) and microbial interspecific associations in shaping moose gut communities while accounting for underlying spatial autocorrelation in microbial occurrences. Accounting for spatial structure not only reduces false detection of interspecific associations that may arise due to dispersal limitation or diet but quantifies how important these processes could be in shaping microbial distributions. Specifically, we address the following questions:

1. Do CRF model combinations including host characteristics and spatial proximity outperform models reflecting just associations between microbes in explaining microbial communities?
2. After controlling for host characteristics and spatial patterns, are there any remaining non-random negative or positive associations between microbes?
3. If there are non-random associations, are they between microbes that are functionally or phylogenetically similar?

We hypothesized that host characteristics would be the dominant factors shaping the moose gut microbial community and that spatial proximity between hosts and interspecific associations would also partially explain gut microbial co-occurrence.

2 | MATERIALS AND METHODS

2.1 | Sample collection and sequencing

Faecal samples were collected from 55 wild moose that were part of companion studies of survival and cause-specific mortality led by the Minnesota Department of Natural Resources, Grand Portage Band of Lake Superior Chippewa and Voyageur's National Park across north-eastern Minnesota (Figure 1), placed on ice until transportation to the laboratory and stored at −20°C prior to processing. This included moose that were live-captured (n = 52, 2011–2015) and sick (n = 3, 2009–2010). Details of the capture and handling of these moose can be found in Butler, Carstensen, Hildebrand, and Giudice (2012) and Carstensen et al. (2018). The metadata provided from these moose included date and location of capture, pregnancy status, sex, age, body condition and serological exposure to *Borrelia burgdorferi*, West Nile Virus and *Leptospira* (six serovars including *L. bratislava*, *L. canicola*, *L. grippotyphosa*, *L. hardjo*, *L. icterohemorrhagica* and *L. pomona*). In total, we had serological data for 49 moose (33 females and 16 males) with the remaining six individuals removed from the CRF analysis (but retained for the descriptive analysis). See Butler et al. (2012) for serological test details. See Table S1 for examples and of how each pathogen could potentially impact gut microbial communities.

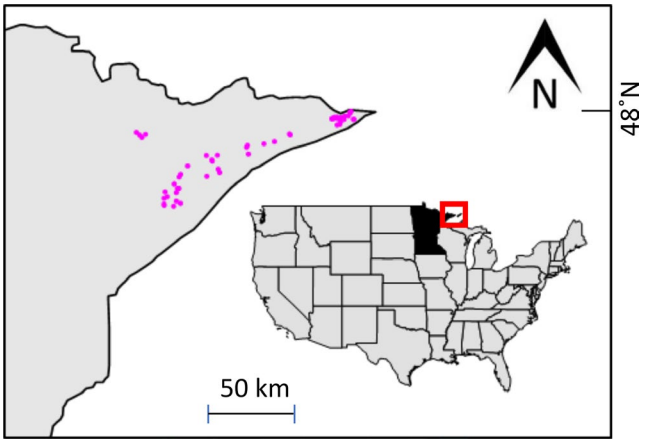

FIGURE 1 Locations (pink dots) of moose faecal samples in north-eastern Minnesota, USA. The red box shows the location of the study in Minnesota

Of the live-captured moose, 21 were observed to be underweight (thin/very thin), and 31 were considered of normal body condition at winter capture (January–February). All samples apart from one were collected in winter between 2011 and 2015; the other sample was collected in 2003. Microbial DNA was extracted from the faecal samples using the PowerSoil DNA Isolation Kit (Qiagen), in accordance with the manufacturer's protocol. The V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the barcoding primers 515F and 806R (Caporaso et al., 2011). Amplicons were sequenced on the Illumina MiSeq platform following the method outlined by Gohl et al. (2016).

2.2 | Bioinformatics pipeline

Raw sequencing reads were processed using the University of Minnesota's metagenomics pipeline (a complete description of the pipeline can be found at https://bitbucket.org/jgarbe/gopher-pipelines/wiki/metagenomics-pipeline.rst), which implements the QIIME version 1.9.1 analysis software (Caporaso, Bittinger, et al., 2010; Caporaso, Kuczynski, et al., 2010). First, each fastq file was reduced to the lowest number of reads in any sample (101, 131) in order to reduce processing time. Sequencing adaptors were then trimmed

using Trimmomatic (Bolger, Lohse, & Usadel, 2014), read pairs were assembled, and primer sequences were removed using PANDAseq (Masella, Bartram, Truszkowski, Brown, & Neufeld, 2012). Reads without primers, unpaired reads and assembled reads that were outside the expected rRNA gene V4 region length were discarded. Chimeras were detected and removed with QIIME's identify_chimeric_seqs.py function with the usearch61 algorithm (Edgar, 2010). Open-reference OTU (operational taxonomic unit) picking was conducted using QIIME's pick_open_reference_otus.py with a minimum sequence identity threshold of 97%. Representative OTU sequences were aligned against the Greengenes version 13_8 core set (DeSantis et al., 2006) using UCLUST (Edgar, 2010) with QIIME default parameters. Singleton OTUs, those that did not align with PyNAST (Caporaso, Bittinger, et al., 2010; Caporaso, Kuczynski, et al., 2010), and those that were classified as chloroplast or mitochondrial DNA were removed from the analysis. The final OTU table was then rarefied to 100,859 reads per sample to normalize our data and ensure that read depth did not bias results.

To test for co-occurrence patterns between gut microbes, we filtered out OTUs with ≤10% prevalence (i.e. only keeping OTUs that occurred in at least 10% of samples) and converted the OTU table into a presence–absence matrix. OTUs that were found ≥75% of samples were also filtered from the analysis. Our purpose for removing rare and common OTUs was to ensure that adequate inferences could be made about the occurrence probability of each OTU (Ovaskainen, Abrego, Halme, & Dunson, 2016). This would be impossible to do if the target OTU shows little variability across sampled environmental gradients by being either too rare or too common. These particular cut-off values of 10%–75% were chosen because initial model testing revealed that the strong class imbalances for OTUs outside these thresholds hampered the ability of our cross-validation procedures to estimate regression coefficients (i.e. a cross-validation training fold that contains nearly all 0s or all 1s is not informative for estimating effects of predictors and will result in an intercept-only model instead, Galar, Fernandez, Barrenechea, Bustince, & Herrera, 2012). We transformed the presence–absence matrix into a Jaccard similarity matrix and used non-metric multidimensional scaling (nMDS) to select OTUs that were driving compositional change across the moose samples and therefore likely to differ with moose characteristics such as body condition (Pearson correlation coefficient [ρ] ≥ 0.25 with any axis). We selected how many axes to include and goodness-of-fit of the nMDS analysis by examining stress (values <0.2 indicate a reasonable representation of community composition) and visual inspection of Shepard diagrams. In total, a reduced set of 43 OTUs remained after this filtering process. To test how sensitive our models are to this threshold, we reduced the nMDS threshold to $\rho \geq 0.2$ and performed the following analysis again. Patterns of OTU composition were not further explored. NMDS was performed using functions in the R package VEGAN (Oksanen et al., 2013).

To reduce the dimensions of the serological exposure data for subsequent analysis, we converted test results into a binary matrix (0 if the individual tested negative and 1 if positive), transformed the binary matrix into a Jaccard similarity matrix and then performed principal coordinate analysis (PCoA) using the R package VEGAN (Oksanen et al., 2013). The first three PCoA axes represented 85% of the variation in exposure across individuals, and eigenvalues from these axes were used as covariates in the CRF analysis below (now called *pathogen exposure PCoA axes*).

2.3 | Conditional random fields

2.3.1 | Identifying OTU co-occurrence patterns using CRFs

The framework we used to investigate OTU co-occurrence probabilities while accounting for potential influences of covariates is described in detail by Clark, Wells, and Lindberg (2018a) and references therein. Briefly, the log-odds of observing OTU *j* given covariate *x* and the presence–absence of OTU *k* is modelled using:

$$\log\left(\frac{P\left(y_j=1|y_{\setminus j},x\right)}{1-P\left(y_j=1|y_{\setminus j},x\right)}\right)=\alpha_{j0}+\beta_j^T x+\sum_{k:k\neq j}\left(\alpha_{jk0}+\beta_{jk}^T x\right)y_k, \quad (1)$$

where y_j is a vector of binary observations for OTU *j* (1 if the OTU was present, 0 if absent), $y_{\setminus j}$ represents vectors of binary observations for all other OTUs apart from *j*, α_{j0} is the OTU-level intercept, and β_j^T is the coefficient for the effect of covariate *x* on OTU *j*'s occurrence probability. Interaction parameters are represented by α_{jk0} and $\beta_{jk}^T x$ (defined below). Parameterization of the likelihood is estimated using logistic regression, where regression coefficients represent the effects of predictors on the OUT's conditional log-odds. Cross-multiplying all combinations of co-occurring OTUs and external covariates allows direct comparison of the relative influences of interspecific associations and host effects on an OUT's occurrence probability. For each OTU-specific regression, sparsity is added to the model using L1 (e.g. least absolute shrinkage and selection operator [LASSO]) regularization to force regression coefficients towards zero if they have minimal effects. Ten-fold cross-validation was implemented to choose the penalty that minimized cross-validated error, as this is considered an appropriate loss function in binomial classification studies. We repeated this process up to 50,000 times for each regression (with a new random cross-validation split chosen for training in each iteration), ensuring that the data were appropriately covered during model fitting. Following LASSO variable selection, coefficients representing conditional dependence of two OTUs and coefficients representing effects of covariates on this dependence were symmetrized by taking the mean of the corresponding estimates so that $\alpha_{jk0} = \alpha_{kj0}$ and $\beta_{jk}^T x = \beta_{kj}^T x$. This means we can approximate parameters from a unified graphical network, after maximizing the conditional log-likelihood for each OTU (Lee & Hastie, 2015). Following unification, inference is straightforward. If $\alpha_{jk0} = 0$, we can infer that the occurrence probabilities of OTUs *j* and *k* are conditionally independent, after accounting for covariates

and other OTUs. If $\alpha_{jk0} \neq 0$ but $\beta_{jk}^{T}x = 0$, the occurrence probabilities are still considered conditionally dependent, but the strength of this dependence is not expected to covary with covariate *x*.

2.3.2 | Markov random field and CRF model selection

We estimated four graphical model formulations of increasing complexity to identify a best-fitting model for our OTU presence–absence dataset. In general, the Markov random field (MRF) models do not include covariates, while the CRF models included both the covariates and covariate by OTU interactions as additional predictors. Furthermore, we performed MRF and CRF routines with and without spatial regression splines giving us a total of four separate models. In the first, we built a MRF graph that did not include any spatial data or host characteristics and used only the binary occurrences of the 43 OTUs as predictors (hereafter the *MRF model*). In the second model, the GPS coordinates for each observation (latitude and longitude, in decimal degrees) were used to construct penalized Gaussian process regression splines with 100 degrees of freedom (Kammann & Wand, 2003; Wood, 2003; hereafter the *spatial MRF model*). Including the spatial splines in each OUT's linear predictor ensured that interspecific associations on occurrence probabilities were estimated only after accounting for possible spatial autocorrelation. For the third model, we built a CRF including moose characteristics covariates (moose sex, body condition, pregnancy status and the pathogen exposure PCoA axes) as well as longitude and latitude (hereafter the *non-spatial CRF model*). All covariates were included as scaled continuous variables with the exceptions of sex and pregnancy status, which were both included as categorical variables. For the final model, we built a CRF as above, but we replaced the latitude and longitude variables with spatial splines (hereafter the *spatial CRF model*).

We assessed the fit of each of the above candidate models to the observed data by calculating the proportion of observations that each model successfully classified. This was done using 10-fold cross-validation fitting models to a random fold containing 90% of the data (training data) and predicting OTU occurrence for the remaining 10% (test data). The best-fitting model was then fit to 100 bootstrapped versions of the observed data (randomly shuffling observations in each bootstrap iteration) to capture uncertainty in model parameters. All CRF and MRF model fitting was performed using functions in the MRFcov R package (Clark et al., 2018a). From the OTU co-occurrence data, we constructed an adjacency matrix and plotted association networks using iGraph R package (Csárdi & Nepusz, 2006). See Appendix S1 for R code detailing data preparation, analytical routine and model specification.

As an additional sensitivity analysis, and to gain further insight into the utility of multivariate community information for predicting changes in microbiota composition, we fit 'null' models that only included spatial effects (Gaussian process spatial splines, as in the spatial models above) as well as host- and site-level predictors. In other words, these null models did not include the presence/absence of other OTUs as predictors. For each OTU null model, we built LASSO logistic regressions that used 10-fold cross-validation as above to identify appropriate LASSO penalties. We predicted binary occurrences using the resulting model fits and calculated predictive metrics as above for the entire dataset (2,184 observations).

2.4 | OTU functional predictions

We predicted the molecular functions of OTUs using PICRUSt's precalculated functional prediction table, where the rows were KEGG orthologs (KOs) and the columns were OTUs based on Greengenes identification numbers (DeSantis et al., 2006; Kanehisa, Sato, Kawashima, Furumichi, & Tanabe, 2016; Langille et al., 2013). This KO table was converted into a presence–absence matrix (i.e. whether a particular functional ortholog is associated with that OTU) and calculated the pairwise functional similarity using the Jaccard index. We applied nMDS and unweighted average linkage clustering to view the broad functional relationships between OTUs and define functional groups (FGs).

3 | RESULTS

We found that Firmicutes, followed distantly by Bacteroidetes, were the dominant phyla in all moose gut communities, together making up over 85% of total reads (Figure S1). The ratio of these groups across samples was relatively consistent (Figure S2), with 81% (±3.5%) mean abundance for Firmicutes and 6.9% (±2.4%) for Bacteroidetes. The reduced set of 43 OTUs used to test for co-occurrence patterns had relatively low abundance across all samples making up on average only 0.32% (±0.37%) of the microbial community (Figure S3). These OTUs were primarily of the classes Clostridia and Mollicutes, within the phyla Firmicutes and Tenericutes, respectively (Table 1; Figure S3). Figure S4 illustrates how important this reduced set of OTUs was in explaining overall community composition (strong OTU correlations with each axis). Comparison of functional orthologs between these OTUs suggested two main FGs, with Firmicutes and Tenericutes OTUs in separate groups (Figure S5).

3.1 | Model fit

The MRF model, which only quantified associations between microbes, could more accurately predict OTU occurrence compared to either the spatial CRF or non-spatial CRF models (both of which included host characteristics; Figure 2a). Including spatial regression splines did improve the fit of the CRF model, with this model accurately predicting 78% of observations compared to 74% of for the non-spatial CRF (Figure 2a). In contrast, the MRF could

TABLE 1 Summary of associations detected in the Markov random field analysis

OTU identity	Avg.oc	Pos	Neg	Phylum: Class: Order	FG
otu206930	0.3466	5	4	Cyanobacteria:4C0d-2:YS2	a
otuNewReferenceOTU123	0.4046	3	2	Cyanobacteria:4C0d-2:YS2	N
otu129755	0.4823	2	0	Firmicutes:Clostridia:Clostridiales	A
otu17822	0.0133	7	1	Firmicutes:Clostridia:Clostridiales	a
otu266952	0.7488	5	4	Firmicutes:Clostridia:Clostridiales	a
otu291630	0.4244	4	0	Firmicutes:Clostridia:Clostridiales	a
otu294064	0.4212	5	0	Firmicutes:Clostridia:Clostridiales	a
otu294262	0.2985	4	0	Firmicutes:Clostridia:Clostridiales	a
otu296918	0.8797	1	8	Firmicutes:Clostridia:Clostridiales	a
otu322906	0.9473	0	5	Firmicutes:Clostridia:Clostridiales	a
otu325706	0.4391	5	2	Firmicutes:Clostridia:Clostridiales	a
otu333577	0.2737	3	1	Firmicutes:Clostridia:Clostridiales	a
otu4295783	0.3602	4	2	Firmicutes:Clostridia:Clostridiales	a
otu4314603	0.5133	2	4	Firmicutes:Clostridia:Clostridiales	a
otu4417708	0.3572	5	7	Firmicutes:Clostridia:Clostridiales	a
otu561193	0.4458	6	2	Firmicutes:Clostridia:Clostridiales	a
otu574585	0.5554	1	4	Firmicutes:Clostridia:Clostridiales	a
otu579159	0.1219	5	2	Firmicutes:Clostridia:Clostridiales	a
otu590209	0.1337	4	1	Firmicutes:Clostridia:Clostridiales	a
otu738351	0.6364	1	0	Firmicutes:Clostridia:Clostridiales	a
otu99718	0.601	4	2	Firmicutes:Clostridia:Clostridiales	a
otuNewCleanUpReferenceOTU157	0.0953	4	0	Firmicutes:Clostridia:Clostridiales	N
otuNewCleanUpReferenceOTU76	0.1108	5	3	Firmicutes:Clostridia:Clostridiales	N
otuNewReferenceOTU108	0.2242	6	1	Firmicutes:Clostridia:Clostridiales	N
otuNewReferenceOTU268	0.479	2	0	Firmicutes:Clostridia:Clostridiales	N
otuNewReferenceOTU74	0.2765	1	0	Firmicutes:Clostridia:Clostridiales	N
otuNewReferenceOTU76	0.4206	6	5	Firmicutes:Clostridia:Clostridiales	N
otu4396877	0.2037	2	0	Firmicutes:Erysipelotrichi:Erysipelotrichales	a
otuNewCleanUpReferenceOTU345	0.0006	1	0	Firmicutes:Erysipelotrichi:Erysipelotrichales	N
otuNewCleanUpReferenceOTU42	0.5424	4	5	Firmicutes:Erysipelotrichi:Erysipelotrichales	N
otu4322320	0.005	4	0	Proteobacteria:Betaproteobacteria:Burkholderiales	a
otu311535	0.1033	6	0	Tenericutes:Mollicutes:Anaeroplasmatales	b
otu292967	0.0006	1	0	Tenericutes:Mollicutes:RF39	b
otu317385	0.1731	5	1	Tenericutes:Mollicutes:RF39	b
otu513605	0.2521	6	2	Tenericutes:Mollicutes:RF39	b
otu531589	0.5759	6	1	Tenericutes:Mollicutes:RF39	b
otu921020	0.2482	4	1	Tenericutes:Mollicutes:RF39	b
otuNewCleanUpReferenceOTU34	0.0806	5	2	Tenericutes:Mollicutes:RF39	N
otuNewReferenceOTU390	0.3596	6	0	Tenericutes:Mollicutes:RF39	N
otuNewReferenceOTU404	0.0469	5	3	Tenericutes:Mollicutes:RF39	N
otuNewReferenceOTU413	0.1843	6	1	Tenericutes:Mollicutes:RF39	N
otuNewReferenceOTU493	0.1678	5	1	Tenericutes:Mollicutes:RF39	N
Average		4	2		

Note: Avg.oc: average occurrence in our moose samples. Pos: number of positive associations. Neg: number of negative associations. 'NewReference' indicates that the OTU has not been previously characterized by the Greengenes database. FG: functional group (see Figure S5). N: no functional group could be determined (not previously characterized).

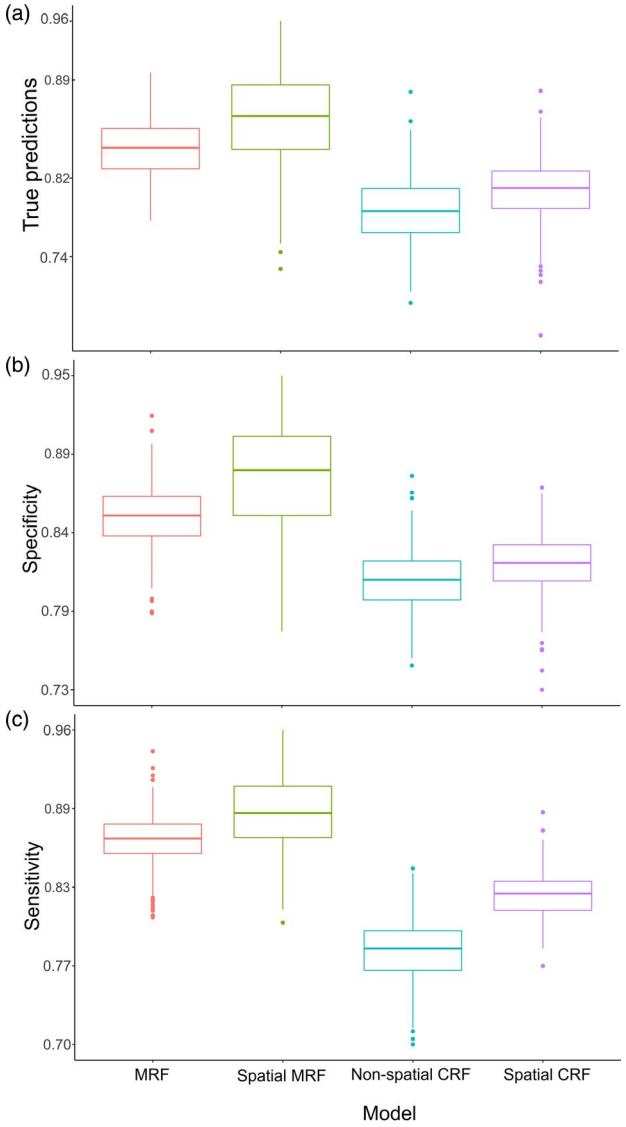

FIGURE 2 Box-and-whisker plots showing the predictive performance of the Markov random field model (MRF—without model covariates), spatial MRF model, non-spatial conditional random field model (CRF—with model covariates) and spatial CRF as defined by (a) true prediction performance, (b) specificity and (c) sensitivity. Predictions were assessed by fitting models to a random fold containing 90% of the data (training data) and predicting operational taxonomic unit (OTU) occurrence for the remaining 10% (test data). This process was repeated 100 times to capture uncertainty in each model. Specificity is the ability of the model to correctly identify individual moose without the specified OTU (proportion of observed negatives that were predicted to be negative), while sensitivity is the ability to correctly identify individuals with the specified OTU (proportion of observed positives that were predicted to be positive). Box hinges show the interquartile range (25% and 75% quantiles), lines within boxes indicate the median (50% quantile), whiskers show 10% and 90% quantiles, and dots show values outside these quantiles

accurately predict 83% of presence–absence observations on average (Figure 2a). This result was not due to one model being better able to predict occurrence over absence, as the MRF had much higher specificity and sensitivity than either of the CRF models (Figure 2b,c). However, we still found evidence of spatial clustering in microbial occurrence probabilities, as MRF performance improved further when we included spatial splines in the model (Figure 2a). Adding spatial data to this model enabled us to correctly predict ~90% of the OTU occurrences for our subset of the moose gut microbial community. This not only suggests that microbes in our dataset were more likely to show similar occurrences in moose that were sampled nearby to one another, but that false associations between microbes could be inferred by models that do not account for spatial proximity. Reducing the nMDS correlation threshold to $\rho \geq 0.2$ included 98 OTUs in the analysis, yet the results were similar (see Appendix S2 for details). As the models including 43 OTUs were easier to visualize, we present those results herein.

The null models, which only included spatial effects as well as host- and site-level predictors (e.g. did not include other OTUs as predictors), had substantially lower predictive power than models that included microbial interspecific associations. For the null model, the proportion of true predictions was 67.3%–71.3%. This drop in performance was primarily due to a loss of sensitivity (65.0%, compared to 78% for the worst-performing community model; Appendix S1; Figure 2). Specificity for the null model was on par with the CRF models at 73.3%, but this was still much lower than the MRF models (Appendix S1; Figure 2).

3.2 | Microbial associations

The co-occurrence network revealed that both positive and negative associations occurred across taxonomic and functional groups with no clear phylogenetic pattern (i.e. OTUs from the same phyla/class/order or function were not preferentially negatively or positively associated with each other, Figure 3). We did not include genus-level information as this information was not known for all OTUs. On average, we found that positive associations between OTUs were more common than negative associations (4 vs. 2, Table 1). Firmicutes was the best-represented phyla in our moose faecal samples (Figure S1), and this phylum also had the highest number of OTU associations. Strikingly, even though Bacteroidetes was the second most dominant phyla, we did not detect any associations involving OTUs from these taxa. In contrast, OTUs from Tenericutes were rare (3% of sequences, Figure S1), but overall, they had on average 1 more positive association partner than other phyla (4 vs. 3), whereas Cyanobacteria OTUs had more negative association partners than other phyla (3 vs. 2, Table 1). FGs showed similar differences, with FGb (which was dominated by the Tenericutes; Figure S5) having more positive associations to FGa (5 vs. 4 on average). The opposite was true for negative associations, with FGa having more associations on average than FGb (1 vs. 2). Overall, an OTU from class Clostridia (Firmicutes) had the highest number of associations overall (11, Table 1). OTUs from this class also had the highest number of positive and negative associations (7 and 8, respectively, Table 1).

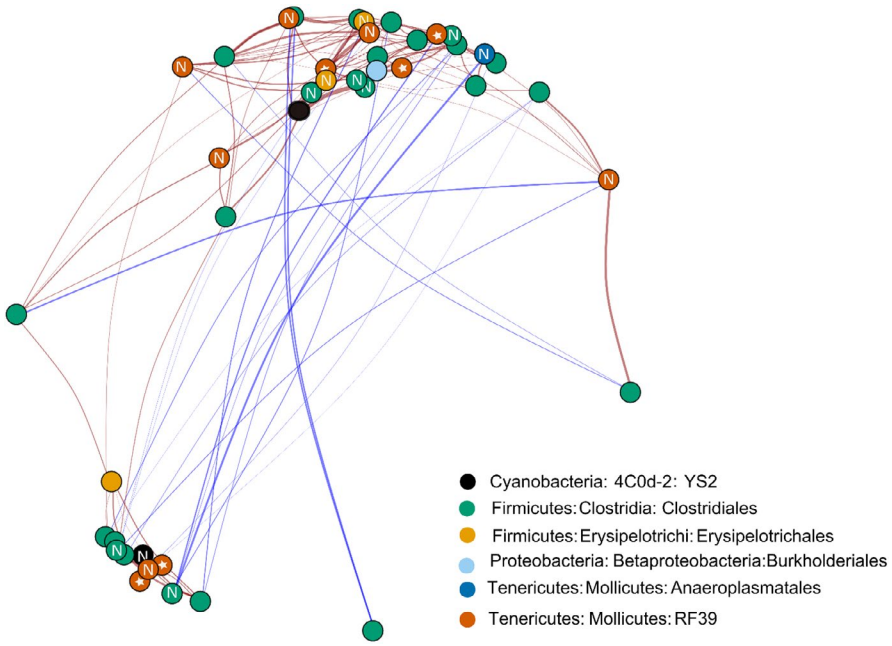

FIGURE 3 Moose gut microbial Markov random field co-occurrence network where nodes closer in space have stronger positive associations with each other. Blue edges indicate negative associations, and red edges represent positive associations. Edge thickness is scaled by the strength of association. Node colour indicates taxonomic group of each microbe. Stars on the node reflect operational taxonomic units (OTUs) from FGb (nodes with no stars cluster with FGa, see Figure S5). 'N' indicates that there were no functional data for this microbe. See Figure S6 for the OTU correlation matrix with names included

4 | DISCUSSION

Utilizing graphical network models, we show the importance of microbial interspecific associations over some host characteristics in shaping moose gut microbial communities at a population scale. In this case, both interspecific associations anspatial proximity were important for shaping microbial communities in this declining moose population. In contrast, the host characteristics we measured were relatively less important in predicting the distribution of microbes. Even though we did not have host genetic or specific diet data from individuals (i.e. stable isotope data), we could predict microbial distributions using just interspecific associations with high accuracy. Across this moose population, we detected non-random negative and positive microbial associations with no clear functional or phylogenetic pattern further supporting transmission as a shaping factor in microbial communities. Our study not only highlights the importance of accounting for interspecific associations when trying to quantify how host characteristics shapes host microbial community but also shows the value of graphical network models in untangling community dynamics more broadly.

We found that evidence of pathogen exposure was not particularly important in predicting moose gut microbial community dynamics. As the serological evidence we used in this study only infers past infection by a particular pathogen rather than current infection status, perhaps this is not surprising. Previous studies testing the role of pathogens shaping microbial communities have used evidence of current infection (e.g. qPCR; Dietrich, Kearney, Seamark, Paweska, & Markotter, 2018; Zink et al., 2015) rather than previous exposure based on serological evidence. When we sampled the moose gut microbial communities, the animal may not be experiencing the infection, and this may be the reason we did not detect an effect. It is also possible that herbivore gut microbial communities are more resilient to pathogen infection than other trophic groups such as carnivores. Ruminant microbial communities are more dominated by bacteria cultivated by the host to aid digestion (Muegge et al., 2011) and may be less regulated by the immune system compared to the gut microbial communities in other species (Ley et al., 2008). Lower immune regulation, for example, could explain why the herbivores such as the gorilla *Gorilla gorilla* have microbiomes remarkably resilient to Simian immunodeficiency virus infection compared to omnivorous chimpanzees *Pan troglodytes* or for HIV infection in humans (Moeller et al., 2015). In support of this, we find that across individual moose in our study, the relative proportions of gut microbial taxa were relatively consistent, despite large differences in body condition and exposure status (Figure S2). Similar ratios of the different microbial taxa in moose have also been reported in moose from the east coast of North America (Ishaq & Wright, 2012) even though samples were collected from moose in a different season. Further studies where current pathogen infection status is known in moose and other herbivores are needed to resolve this question.

Interspecific associations, in contrast, were much better predictors of moose gut microbial communities. In free-living communities, the role of interspecific associations in shaping the distributions of species is increasingly recognized (e.g. Aragón, Carrascal, & Palomino, 2018; de Araújo et al., 2014). However, the importance of interspecific associations is likely to decrease with geographic scale (Araújo & Rozenfeld, 2014). Whether this applies to gut microbial communities, in contrast, is poorly studied. In our case, host effects may have been detectable if we sampled a greater number of individuals across a larger portion of moose home range in central North America. Nonetheless, the scale at which wildlife surveillance is performed (and thus faecal samples collected for gut microbiome analysis) is often similar across studies (e.g. Cheng et al., 2015). In general, understanding the role that scale plays in structuring gut

microbial communities, beyond the population scale, is a major challenge for microbial ecology (Antwis et al., 2017; Camp, Kanther, Semova, & Rawls, 2009). Based on our findings, we suggest that larger geographical and/or temporal scales are needed to detect possible impacts of host characteristics on the structure of gut microbial communities.

Even without accounting for host traits such as host genetics that are thought to be important in shaping the gut microbial communities in other herbivores (Kohl, Varner, Wilkening, & Dearing, 2018), our models had high predictive performance. It is possible that our results are more general and that host traits are not important drivers of gut microbial community dynamics. When we increased the number of OTUs, we included in the model the relative importance of interspecific associations compared to host traits did not vary (Appendix S2). However, our results may be due to samples coming from a relatively well-mixed population, even though our moose samples were taken over a large area spanning a maximum of ~150 km. Central North America (including Minnesota) has the highest genetic diversity of all North American moose populations (Hundertmark, Bowyer, Shields, & Schwartz, 2003), but to what extent our samples come from genetically distinct populations is unknown. Further, the host traits we quantified were far from exhaustive and other host physiological traits, such as those associated with host metabolism, may also be important in shaping microbial communities (e.g. Maldonado-Gómez et al., 2016). Future work incorporating host genetics and a more comprehensive set of moose physiological measures may help better understand the role of host characteristics, compared to interspecific associations or transmission in shaping host microbial communities.

Our capacity to predict the moose gut microbial community at the population scale increased when we included spatial information, indicating that either stochastic events such as microbial dispersal, host social structure or spatial similarities in diet may be important in structuring associations between gut microbes. Dispersal of microbes is thought to be passive (Nemergut et al., 2013); however, these microbial species are often in high abundance and have broad distributions making dispersal challenging to quantify (Evans et al., 2017; Zhou & Ning, 2017). Biogeographical studies have shown that dispersal limitation is important for structuring microbial communities including those of the gut (Evans et al., 2017; Hanson, Fuhrman, Horner-Devine, & Martiny, 2012; Moeller et al., 2017), but rarely over a relatively small area within a host population. What role moose diet explicitly played in shaping our moose gut microbial communities is still an open question. Future studies linking host microbial data to measures of host diet, such as stable isotope analysis (Hofman-Kamińska, Bocherens, Borowik, Drucker, & Kowalczyk, 2018), will enable dispersal limitation and diet to be decoupled in future models. Nonetheless, we show that including host spatial data in future work is necessary for robustly quantifying microbial co-occurrence patterns.

We also found strong positive and negative associations between OTUs from different phyla and divergent functional groups in the moose gut microbial community. Clostridia (Firmicutes) and Mollicutes (Tenericutes) were network 'hubs' involved in a relatively high number of positive associations in our moose population, and often with each other (Table 1). For example, it is possible that representative taxa from both groups are either directly facilitative via resources (i.e. compounds derived by one group facilitate colonization by the other: Kaiko & Stappenbeck, 2014), or via immune regulation (i.e. OTUs that provide complementary nutrients to the host are retained by the immune system: de Medina, Ortega-González, González-Pérez, Capitán-Cañadas, & Martínez-Augustin, 2013). Clostridia OTUs are also hubs for associations in the human gut community (Banerjee, Schlaeppi, & van der Heijden, 2018; Faust et al., 2012), but the importance of Mollicutes has not been, to our knowledge, reported elsewhere. Moreover, we detected no negative associations between Bacteroidetes and Firmicutes even though Bacteroidetes was common in our samples; this group may not play a dominant role in structuring moose gut microbial communities as they do in humans (Banerjee et al., 2018; Faust et al., 2012). Whether the same relationships apply in herbivore populations where Bacteroidetes is dominant, as was the case in an Alaskan and Swedish moose (Ishaq & Wright, 2014; Svartström et al., 2017), remains an open question.

Negative associations between pathogens were less frequent, but also important in structuring moose microbial communities. For example, the negative association between two functionally similar Firmicutes OTUs (574,585 and 738,351, see Figure S5b) could represent a competitive interaction, but further experimental work is required to explore this idea. More generally, Faust et al. (2012) found an increased likelihood of negative associations between OTUs that were functionally and phylogenetically dissimilar (with opposite true for more related OTUs). We did not see such a pattern in our co-occurrence network and why we would get such a different result in moose is unclear. The Faust dataset consisted of 240 individuals from the Human Microbiome Project (Methé et al., 2012), but large methodological differences make direct comparison difficult, as factors such as spatial relationships between subjects were not quantified. How robust these relationships are also likely to be impacted by taxonomic resolution of each network (Faust et al., 2012). As we used 16S rRNA gene sequencing, we were unable to get sequence identifications to species level. Further, functional profiles were based on predictions from reference genomes, not direct identification of functional genes or proteins (Langille et al., 2013). Shotgun metagenomic sequencing could allow for both classification sequences to species level and provide much more detailed insights into the functional patterns shaping microbial species associations.

Here, we have demonstrated that graphical network models can untangle how interspecific interactions can shape gut microbial communities in moose. Additionally, we show that MRF and CRF models can robustly construct co-occurrence networks (Clark et al., 2018b) and, coupled with taxonomic and functional information, find high-resolution insights into interspecific associations. Graphical network models, as with other correlation-based approaches, do have limitations. Our approach is currently based on presence–absence of an OTU as the response variable, and incorporating other community

properties, such as species abundance, will further strengthen this methodology and improve our inference on microbial community dynamics. Further, correlations identified by techniques such as MRF or CRF should be treated with caution as they do not imply causation (e.g. Barner et al., 2018; Dormann et al., 2018). Follow-up analysis with tools such as structural equation modelling (SEM) could be used to go beyond correlations to explore potential causal relationships between microbes (Banerjee et al., 2018). Nonetheless, by explicitly incorporating spatial data and covariates into graphical models, our method offers a step forward in characterizing associations between species, and we envisage this method will be broadly useful for researchers working on micro- and macro-community dynamics alike. Studies analysing associations between microbial species are rare in wildlife, even though they can provide important insights into the ecological dynamics operating within or on the host. Given the decreasing cost of microbial surveys and analytical advances such as ours, studies that can disentangle microbial community dynamics in wildlife species will become more frequent, and this can ultimately provide a more nuanced understanding of wildlife health.

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AUTHORS' CONTRIBUTIONS

N.M.F.-J., M.C., T.M.W., J.F., T.J.J., A.C.K. and M.E.C. came up with the project design. N.M.F.-J., N.J.C. and E.A.M. conducted the analysis. N.J.C. coded the spatial MRF/CRF functions. M.C., T.M.W., S.M. and J.F. provided. M.E.C., T.J.J., J.F. and M.C. got funding for the project. All authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

All code and data are available on GitHub: https://github.com/nfj1380/MRF_microbiome and Dryad Digital Repository: https://doi.org/10.5061/dryad.j9kd51c7k (Fountain-Jones et al., 2019).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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