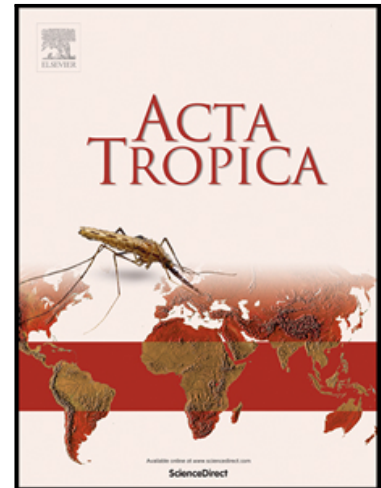


Journal Pre-proof

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Highlights

- Neospora caninum is a major cause of reproductive disorders in dairy cattle farms.
- Bulk tank milk samples were collected from 586 dairy herds in Italy.
- 180 samples were positive for Neospora caninum antibodies (P=30.7%).
- An effect of the parasite on reproductive and productive parameters of the herds was demonstrated.
- A cluster of high risk of infection for Neospora caninum was predicted by spatial scan statistic and Maximum entropy ecological niche modelling.

Journal Pre-proof

***Neospora caninum* antibodies in bulk tank milk from dairy cattle herds in Italy in relation to reproductive and productive parameters and spatial analysis.**

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Abstract

Among the available diagnostic techniques, antibody detection in bulk tank milk (BTM) represents a useful tool to estimate and monitor *Neospora caninum* herd prevalence.

To evaluate the prevalence of *N. caninum* and the effect of parasite infection on herd performances, BTM samples collected from 586 dairy herds located in one of the largest dairy production areas in Italy (Lombardy) were analyzed by an indirect ELISA to detect anti-*N. caninum* specific antibodies. Generalized linear models (GLMs) were developed. A purely spatial analysis scanning for clusters with high or low rates for *N. caninum* using the Bernoulli model was performed. A maximum entropy approach was used to estimate the probability of distribution of the parasite based on occurrence records together with environmental variables.

Overall, 180 herds resulted positive for *N. caninum* antibodies on bulk tank milk (P=30.7%). A higher risk of seropositivity was evidenced in the provinces of Milano, Cremona, Brescia, and Bergamo (P=32-40%); a lower risk was evidenced in Lodi, Pavia, and Mantova (P=13-24%). A higher risk of seropositivity was revealed for small-medium farms (101-300 animals) (O.R.=2.8) and for older animals with more than 4 years (O.R.=4.4).

Regarding the effect of *N. caninum* infection on herd performances, the number of inseminations for conception was higher (> 3 inseminations), and the period from calving to conception was longer (> 150 days) for positive farms (O.R.=2.0 and O.R.=2.3, respectively); besides, lower head daily milk production (<20 kg and 21-25kg) and mature equivalent milk yield (<11000), and somatic cell counts higher than 300000 cells/ml were observed for *N. caninum* positive herds (O.R.=0.4, O.R.=0.4 and O.R.=1.9 respectively).

The geographical distribution of *N. caninum* positive farms with the highest level of probability covers the central sector of the Po Plain where a significant cluster for high risk of parasite infection was shown by spatial scan statistic and Maximum entropy ecological niche modelling. A further significant cluster of low risk occurred in the southern. The climatic and environmental variables with the highest training gain when used in isolation resulted altitude, land use/land cover, and other variables related to temperature and precipitation.

Neosporosis is widely distributed in Italian dairy herds and an impact of the parasite on herd performances could be hypothesized. Even if the role of *N. caninum* in alterations of reproductive and productive parameters should be further explored, veterinarians and farmers should be aware of neosporosis, and control plans should be adopted.

Keywords: Neosporosis; Cows; Herd; Serology; Milk; Abortion.

1. Introduction

Neospora caninum, an obligate intracellular protozoan parasite, is a major cause of abortion in cattle worldwide (Dubey, 2003). Domestic dogs and wild canids (grey wolves, coyotes, and dingoes) are the definitive hosts shedding unsporulated oocysts in their feces; ruminants, equids, and swine can act as intermediate hosts of the parasite (Dubey, 2003; Almería, 2013; Villa et al., 2018; 2022a). In cattle, the parasite can be transmitted horizontally, by ingestion of food or water contaminated by sporulated oocysts, or vertically, through congenital transmission from an infected dam to her fetus during pregnancy (Dubey et al., 2007). Vertical endogenous transmission is responsible for the maintenance of *N. caninum* in infected herds (Conraths and Gottstein, 2007). Even if, the main clinical manifestation of bovine neosporosis is abortion, *N. caninum* was associated with other reproductive problems and an adverse effect of the parasite in early pregnancy was suggested (Dubey et al., 2006; Villa et al., 2022b). Besides, some studies reported a reduction in milk yield in dairy cattle and reduced weight gain and feed efficiency in beef calves and steers, however these data are still controversial (Dubey et al., 2017). Since to date, no safe and effective chemotherapy for bovine neosporosis, and no vaccine against abortion or *N. caninum* transmission, are available, specific control programs should be implemented in the herd incorporating a cost-benefit calculation considering the expenses of testing and control measures with the benefit of reduced economic losses (Reichel et al., 2013).

In cattle, the serological status to *N. caninum* can be assessed in individual animals through serum, plasma, or milk sampling, or in groups of lactating cows by bulk milk testing (Sekiya et al., 2013; Guido et al., 2016). In dairy herds, antibody detection in bulk tank milk represents a useful tool to estimate and monitor *N. caninum* within-herd prevalence (Schaes et al., 2003; 2004). Indeed, the milk of infected cows contains especially IgG1 isotype specific antibodies against the parasite, transferred from the circulating blood into milk secretions (Gonzales-Warleta et al., 2011). Diagnosis of neosporosis by analysis of bulk tank milk may be particularly suitable since milk samples are routinely obtained by the dairy industry for milk quality testing. Besides the diagnosis

of neosporosis at the herd-level, antibody detection in bulk tank milk could also be used to evaluate the effects of *N. caninum* infection on farm production (Gonzales-Warleta et al., 2011). Even if several studies showed a good correlation of bulk milk results with herd seroprevalence assessed testing individual serum samples, it should be considered that a minimum of 10–15% serologically positive animals are necessary to produce positive bulk milk testing results (Bartels et al., 2005). However, ELISA on bulk milk is considered cost-effective and non-invasive indicator of herd status to be applied for control and surveillance purposes (Guido et al., 2016).

In northern Italy, dairy cattle farming is a relevant industrial sector both from a zootechnical and economic point of view, mostly for the production of milk and DOP (Protected Designation of Origin) products such as Parmigiano Reggiano and Grana Padano cheeses. A few previous studies evidenced both the circulation of *N. caninum* in dairy cattle farms from this area and the genetic diversity of parasite isolates from aborted fetuses (Sala et al., 2018; Regidor-Cerrillo et al., 2020; Villa et al., 2021, 2022b). Considering the relevance of dairy production in the area, a large-scale epidemiological study was planned to evaluate the prevalence of *N. caninum* on bulk tank milk in dairy cattle farms from this part of Italy. A second aim was the evaluation of *N. caninum* seroprevalence in association with selected variables including geography, animal attributes, herd size and herd performances. Finally, a spatial analysis scanning for clusters with high or low rates for *N. caninum* with the combination of the influence of climatic and environmental variables to estimate the probability of distribution of the seropositivity to the protozoan infection was assessed.

2. Materials and Methods

2.1. Area description

All farms were located in a geographical area of northern Italy named “Bassa Padana” in the Po Plain in Lombardy. Precisely, they were included in the territory of the administrative districts of Bergamo (BG), Brescia (BS), Cremona (CR), Lodi (LO), Mantova (MN), Milano (MI), and Pavia

(PV) provinces. This is one of Italy's largest dairy production areas and stands out for the high density of cattle farms, mainly based on Italian Holstein Friesian under the intensive production system. The Italian National Zootechnical Registry (https://www.vetinfo.it/j6_statistiche/#/) counts 5274 dairy farms hosting 1,118,998 animals in the Lombardy region, corresponding to the 21.5% and 42.5% of all farms and animals in Italy, with an annual production of 5,318,112 tons of milk (https://report.assolatte.it/2019/Appendice_Statistica_Rapporto2019.pdf).

According to the Köppen-Geiger classification (Kottek et al., 2006; Peel et al., 2007), the climate of the Lombardy region is humid subtropical, with hot, muggy summers, frequent thunderstorms and mild winters with precipitation from mid-latitude cyclones. The average temperature of the warmest month is above 22°C, whereas the average temperature of the coldest month is below 18°C but above -3°C. Considering specifically the Po Plain, the climate is humid subcontinental: in this area, significant variations were observed with respect to the Köppen model for the winter season, due to the presence of thick fog between October and February. The average total annual rainfall in the Lombardy region is 853 mm and, in the Po plain, the pluviometry is slightly bimodal with an absolute minimum in winter, the second lowest values occurring in July, and the two maxima values in November and April (Fazzini et al., 2020).

2.2. Sample and data collection

Bulk tank milk samples were collected from 586 dairy herds during routine quality controls with the cooperation of the Regional Breeders Association of Lombardy. Milk samples were refrigerated during transport to the laboratory and then stored at -20°C until serological analyses.

Data on sampled herds were obtained from the managerial farm system Si@lleva (Italian Breeder Association, www.sialleva.it). For each dairy herd, data on geographical localization (municipality and province), herd consistency (number of animals in the herd), and medium age of the cows in the herd, were recorded, and the herds were classified accordingly. In particular, four classes related to herd consistency were identified: small (≤ 100 animals), small-medium (101-300 animals), medium

(301-500 animals), and large (> 500 animals) herds. Further, three herd age classes were recognized: class 1 (≤ 2 years old), class 2 (> 2 years old < 4), and class 3 (≥ 4 years old).

Data concerning the reproduction performance, i.e. the number of inseminations for conception and the period from calving to conception, were collected. Based on the number of inseminations to conception, three classes were identified: class 1 (< 2 inseminations), class 2 (2-3 inseminations), and class 3 (> 3 inseminations); instead, considering the period from calving to conception, two classes were recognized: class 1 (≤ 150 days) and 2 (> 150 days). Regarding production, the daily milk yield (kg of milk per day) and 305-mature equivalent milk yield (305ME) parameters were considered. The 305 ME allows comparisons among the production of cows from the same breed in different environments and stages of their productive live. The calculation is composed of two parts, the estimation of the 305-day actual milk production based on the milk weights recorded during test days and the mature equivalent correction factor (ME Factor). The ME Factor adjusts all cows to the same age, season of calving and lactation length, and also to the different geographic area of the herd (Si@lIEvA, Italian Breeder Association, www.sialleva.it). Finally, milk qualitative parameters (% of fat and protein content in milk and somatic cell count (SSC: number of somatic cells in a millilitre of milk)) were recorded. Then, herds were classified according to the daily milk production in four classes: class 1 (≤ 20 kg), class 2 (21-25 kg), class 3 (26-30 kg) and class 4 (> 30 kg); two classes were considered for 305-mature equivalent milk yield parameter: class 1 (≤ 11000) and 2 (> 11000). Besides, three classes were identified considering somatic cell counts: class 1 (≤ 200000), class 2 (200000-300000) and class 3 (≥ 300000).

2.3. Serological analyses

Milk samples were analyzed using a commercial ELISA kit (ID Screen® *Neospora caninum* Indirect, Innovative Diagnostics, France) validated for the detection of anti-*Neospora caninum* antibodies in serum, plasma, individual and bulk milk in ruminants, following the manufacturer's instructions. Positive and negative control sera provided with the kit were used as controls. For each

sample, the resulting values were calculated by applying the formula supplied in the kit: $S/P\% = \frac{OD \text{ sample} - OD\text{-negative control}}{OD\text{-positive control} - OD\text{-negative control}} \times 100$. Samples with $S/P\% \geq 60\%$ were considered positive.

2.4. Statistical analyses

Seroprevalence to *N. caninum* in bulk tank milk was calculated at the herd level according to the considered categories (Bush et al. 1997). Serological data were further analyzed to determine which variables could be predictors of *N. caninum* infection; the herd was considered as the statistical unit. Generalized linear models (GLMs) with binomial distribution were performed, and the binary outcome (presence/absence of anti-*N. caninum* antibodies) based on serology results was used as the dependent variable. The following variables, i.e. farm province, herd consistency, medium age, reproductive (number of inseminations for conception and the period from calving to conception), productive (daily milk yield, 305ME) and milk qualitative parameters (% of fat and protein content and milk somatic cell count (SCC)) were separately considered and entered in each model as independent variables. Subsequently, all the variables with a P value = 0.05 were inserted in a multivariate model developed through a backward selection procedure (significance level to remove variables from the model = 0.05) based on Akaike information criterion (AIC) values. Statistical analysis was performed using SPSS software (Statistical Package for Social Science, IBM SPSS Statistics for Windows, 25.0, Chicago, IL, USA).

A purely spatial analysis scanning for clusters with high or low rates for *N. caninum* using the Bernoulli model (SaTScan v.10.1, SaTScanTM, USA) was performed. A maximum entropy approach (MaxEnt vers. 3.4.4) was developed to estimate the probability of distribution of the pathogen based on occurrence records together with environmental variables. 19 different climate variables (layers BIO01 - BIO19) were obtained from the website WorldClim (<https://www.worldclim.org/data/index.html>), a set of global climate layers with a spatial resolution of 1 km² for the mapping and spatial modelling in a geographical information system (QGIS vers.

3.24). Besides, three environment variables (DUSAF 6.0., watercourses, elevation) were obtained from the Geoportal of Lombardy (<https://www.geoportale.regione.lombardia.it/>) (Tab. 1). DUSAF is an Italian acronym for “Use Categories of Agricultural and Forest Soil”. It is a land use/land cover database, periodically updated, created in 2000–2001 within a project funded by the Lombardy Region and carried out by the Regional Authority for Services to Agriculture and Forests (ERSAF) with the cooperation of the Regional Agency for the Protection of the Environment (ARPA) (Credali et al., 2011). For version DUSAF 6.0, orthophotos were used (made by AGEA, 1 pixel=0.2m on the ground), with colour aerial photos taken in 2018 and satellite images SPOT6/7 2018 (1 pixel=1.5m on the ground). The DUSAF classification is organised into five hierarchical levels, of which the first three are formed by the classes of the CORINE Land Cover project, which are then increasingly differentiated to the 5th classification level. The first level includes 5 general classes embracing the principal cover types (Artificial areas, Agricultural areas, Forest and semi-natural areas, Wetlands, Water bodies).

A preliminary analysis was performed to verify the correlation between the climate variables. To reduce collinearity and overfitting of the variables in the model, only variables with a Pearson correlation coefficient $<|0.8|$ were considered in the model building (MacNally 2000; Dormann et al. 2013). The goodness of each model is expressed through the average value of the AUC (Area under the curve) of the receiver-operating characteristics curve (ROC) calculated on 10 replications. The resampling method (Jackknife test) was performed to get estimates of variable importance considering one covariate at a time. The test identifies the environmental variable with the highest gain in the model performance when used in isolation. The gain expresses the average probability of the point localities, and it is the log of the number of grid cells minus the log loss (average of the negative log probabilities of the sample locations).

3. Results

Out of 586 bulk tank milk samples, 180 herds resulted positive for *N. caninum* antibodies, with a prevalence of 30.7%. The descriptive results of the prevalence of *N. caninum* infection on bulk tank milk according to the considered herd data are showed in Tab. 2.

Most tested positive herds were located in the provinces of Milano, Cremona, Brescia and Bergamo and the prevalence concerning these subpopulations varied between 32.3 and 41.7%; instead, Lodi, Pavia and Mantova showed lower seroprevalence with values between 13.5 and 24.2%.

The seropositivity to *N. caninum* infection was higher for small-medium herds (P=40%) if compared to small and medium ones (P=24.0 and 28.3%, respectively). A lower positivity level (P=19.2%) was recorded for larger herds. Overall, the medium consistency of positive herds was lower if compared to negative ones (283.9 and 381.7 animals, respectively).

Concerning the medium age of the animals in sampled herds, herds with older animals (≥ 4 years) and those with cows between 2 and 4 years old resulted more exposed to the parasite infection with a seroprevalence equal to 37.7% and 30%, respectively. On the contrary, a prevalence of 12.0% was evidenced for herds with cows ≤ 2 years old. Despite this, the medium age of positive herds was similar to negative ones (3.3 and 3.1 years old, respectively).

Regarding the herd performances, among reproductive parameters, even if the number of inseminations for conception was almost similar for positive (2.7) than for negative herds (2.5), the prevalence of *N. caninum* was 39.7% for herds with repeat breeders' cows with more than 3 services to conception if compared to those with < 2 and 2-3 inseminations (P=25.0% and 29.3% respectively). Besides, the period from calving to conception was longer for positive herds (173.4) if compared to negative ones (160.4 days); the positivity to the protozoan infection was higher for herds with days open of more than 150 days if compared to those with this period of less than or equal to 150 days (P=38% and 21%, respectively).

Concerning productive parameters, daily milk yield and 305ME were lower for positive herds if compared to negative ones (23.2 kg and 25.6 kg, 9801 and 10152, respectively). The seropositivity

to *N. caninum* infection was more frequently detected in herds with a daily milk production lower than or equal to 20 kg (P=40.2%) and between 21 and 25 kg (P=37.6%), whereas herds producing between 26 kg and 30 kg (P=22.0%) and more than 30 kg (P=20.4%) of milk were less often positive. Besides, a higher presence of antibodies anti-*N. caninum* was detected for herds with 305ME lower than or equal to 11000 if compared to those with this value higher than 11000 (P=37.8 and 22.4%, respectively).

Considering milk qualitative parameters, no differences were revealed in fat and protein content for positive and negative herds (3.80% and 3.82% and 3.39% and 3.38% respectively). Somatic cell count was slightly higher for positive (257602) if compared to negative herds (238221); herds with more than or equal to 300000 cells (P=40.3%) were more frequently tested positive than those with 200000-300000 and less than or equal to 200000 (P=30.6% and 26.0%, respectively).

Univariate analysis revealed statistically significant associations between the positivity to *N. caninum* infection and herd province, herd consistency, herd medium age, number of inseminations for conception, the period from calving to conception, daily milk yield, 305ME and somatic cell count (Tab. 3). Nevertheless, herd province, herd consistency, the period from calving to conception and 305ME were the only variables entered in the final multivariate model (Tab. 4). Indeed, the province of herd location had impressive effect on the positive status of the herd for *N. caninum* antibodies; particularly, herds from Bergamo, Brescia and Cremona showed highest risk to become positive (OR=6.8, OR=6.1, OR=4.8 respectively). Further, the odds of small-medium herds being positive were 1.7 greater than herds belonging to the other categories related to herd consistency. Regarding herd performances, herds presenting a period from calving to conception shorter than or equal to 150 days were less likely positive for the parasite infection (OR=0.5). On the other hand, the herds with 305ME lower than or equal to 11000 showed higher risk to become positive (OR=2.2) than herds with 305ME >11000.

The geographical distribution of *N. caninum* positive herds revealed that the positive herds were grouped in the central and the western sectors of the Po Plain (Fig.1). However, Bernoulli spatial

analysis only evidenced two significant clusters: one high-risk cluster in the central area with a 50.3% of herds tested positive and a relative risk of 2.13 and one low-risk cluster in the southern area with no seropositive cases (Fig. 1) (Tab.5). The potential geographical distribution of *Neospora*-positive herds, predicted by the MaxEnt program, covered mainly the central sector of the Po Plain (Fig. 2). The model performance developed on the selected climatic and environmental variables was very good (average AUC=0.902±0.008). Interestingly, the two significant high and low-risk clusters for *N. caninum* seropositivity showed by SatScan overlapped with the areas with high and low probability of seropositivity evidenced by MaxEnt model, respectively. Besides, also the group of positive herds in the western area, even if not a significant cluster, overlapped with a high-risk seropositivity area. Altitude (ELEV) achieved the highest training gain when used alone to build the model. Other variables that show training gain when modelling with a single variable included: mean temperature of warmest quarter (BIO10), temperature seasonality (BIO4), use categories of agricultural and forest soil (DUSAF), precipitation seasonality (BIO15), annual precipitation (BIO12), and precipitation of coldest quarter (BIO19) (Fig. 3).

4. Discussion

In this survey, we studied the circulation of antibodies to *N. caninum* in dairy cattle herds in a northern Italian region highly anthropized and with a high density of dairy cattle farms. In comparison with a previous study conducted in Italy on bulk tank milk, the herd prevalence of *N. caninum* antibodies of 30.7% of our survey is lower in Lombardy than in Sardinia Island (55%) (Varcasia et al., 2006). This result is also lower than those obtained by other studies on serum samples in both Italian northern and southern regions, reporting prevalence values between about 40 and 80% (Otranto et al., 2003; Rinaldi et al., 2005). Otherwise, the seroprevalence of *N. caninum* in the 586 sampled dairy herds was higher than the seroprevalence values of 15.5 and 18.5% reported in two dairy herds recruited as a case study from the same area (Villa et al., 2022b). Furthermore, it

should be considered that the prevalence value obtained with the analysis of bulk milk in this study does not exactly represent the actual seropositivity status of the herd as heifers and dry cows were excluded, and therefore the seropositivity value could be higher, also considering the dilution of antibodies in bulk milk if compared to individual samples. Besides, a molecular study in Lombardy recorded a prevalence of 27.8% on bovine aborted fetuses, confirming the involvement of *N. caninum* in reproductive failure in cattle of the area (Villa et al., 2021).

Concerning the European scenario, a pooled seroprevalence of 13% was estimated for *N. caninum* in cattle (Ribeiro et al., 2019). Other studies conducted on bulk tank milk reported a wide range of prevalence values from less than 1% up to 60% in different countries (McCarthy et al., 2021; Klevar et al., 2010; Schares et al., 2009; Hurkova et al., 2005; Schares et al., 2003; Frossling et al., 2008; Sekiya et al., 2013; O'Doherty et al., 2013; Chatziprodromidou et al., 2017; Gonzalez-Warleta et al., 2011). However, it should be considered that the results of these studies may be influenced by experimental study design, different serological methods and cut-off, sample size, farm size, management and exposure to risk factors, and geographical area with regional and climatic variations (Cirone et al., 2021).

The herd consistency showed to have strong effects on *N. caninum* serostatus of cattle herds, resulting in a higher risk for the small-medium size herds (P=40%). Besides, the prevalence was similar for small (P=24.0) and medium (P=28.3%) herds but lower for large ones (P=19.2%). As previously reported, the lower prevalence related to the parasite of the larger, more specialized, and industrialized farms could probably be explained by better hygiene-sanitary conditions and handling practices (Bartels et al., 2006; González-Warleta et al., 2008; Razmi and Barati, 2017). However, smaller familiar herds with less than 100 animals may pay more attention to cows due to the low number of animals. Indeed, general management and biosecurity-related risk factors were demonstrated to be significantly associated with an increased probability for the positivity of bulk milk to *N. caninum* (O'Doherty et al., 2014).

The herd age resulted weakly associated with seropositivity of the herd; indeed, a significant risk of being positive for *N. caninum* antibodies was only observed for herds with a medium age ≥ 4 years. Moreover, a few studies reported higher parasite infection in older animals (Razmi et al., 2006; Gharekhani et al., 2021; Villa et al., 2022b). Furthermore, as already demonstrated, the exposure to *N. caninum* may increase with parity number, number of pregnancies, gestation number, or number of lactations (Eiras et al., 2011). The increase in prevalence with age also indicated the existence of a high probability of horizontal transmission (Bartels et al., 2006). In this regard, apart from the role of the definitive hosts, also the influence of other domestic and wild species acting as intermediate hosts, and thus reservoir of the parasite, should not be neglected. Indeed, the presence of antibodies to *N. caninum* was previously demonstrated in small ruminants (Gazzonis et al., 2020), horses (Villa et al., 2018), pigs (Villa et al., 2022a), wild boars (Villa et al., 2023) and wild birds (Gazzonis et al., 2021) in Lombardy region confirming the environmental circulation of the parasite.

The present study revealed a relation between the herd positivity to *N. caninum* antibodies and the considered reproductive parameters. Indeed, an effect both on the number of inseminations and the calving to conception interval was demonstrated in the univariate analysis. However, as evidenced by the multivariate analysis, *N. caninum* positivity resulted to have a relevant effect only on the calving to conception interval with a value > 150 days. Previously, in two case study herds in Lombardy, in *N. caninum* positive cows, higher number of inseminations (2.4 and 2.9 in seropositive cows vs 2.1 vs. 2.4 in negative ones) and days in milking (167.7 and 221.6 in seropositive cows vs 133.4 and 219.6 in seronegative ones) were evidenced (Villa et al., 2022b). Furthermore, in dairy cattle from Brazil, Cruz et al. (2011) observed a higher mean number of services for conception (3.9) in *N. caninum* seropositive cows if compared to seronegative ones (2.3); these authors also observed a higher value (189 days) of the calving to conception interval in *N. caninum* seropositive cows if compared to seronegative ones (146 days). A higher mean number of artificial inseminations (3.92) and a longer birth interval (199) in seropositive dams if compared to seronegative ones (2.39 and 120, respectively) was reported in northern Greece (Lefkaditis et al.,

2020); the same pattern was also observed in Turkey with 2.29 vs 3.84 inseminations and 120 vs 197 days open in seronegative and seropositive cows, respectively (Canatan et al., 2014). An increase in the number of inseminations for conception was also demonstrated in Australia and USA (Munoz-Zanzi et al., 2004; Hall et al., 2005). As previously suggested, these findings may indicate embryonic death followed by fetal reabsorption, leading to a return to heat, defined as repeat breeders (Kamga-Waladjo et al. 2001, De Barros et al., 2021). However, other studies did not show any influence of the parasite infection on these parameters indicating a reduced fertility in early pregnancy (Jensen et al., 1999; Lopez-Gatius et al., 2004; Brickell et al., 2010; Gonzales-Warleta et al., 2011).

Regarding the influence of the parasite infection on productive performances, in this study both daily kg of milk and mature equivalent milk yield were lower in positive herds. In particular, the positivity to *N. caninum* was related to a daily milk production < 25 kg in the univariate analysis; moreover, the influence of the seropositivity to the parasite on 305ME < 11000 was evidenced in both the univariate and multivariate analysis. Previously, two dairy herds from the same sampling area showed opposite patterns concerning daily milk production and 305ME according to the serostatus of the cows (Villa et al., 2022b). Indeed, the few studies analyzing the effect of *N. caninum* infection on milk production provided contradictory results. A few authors indicated a decrease in milk production in seropositive animals, with a reduction of 1.41 kg/cow/day in first-lactation dairy cows (Thurmond et al., 1997) and 1.27 kg/cow/day regardless of parity (Hernandez et al., 2001). A small detrimental but not significant effect of seropositivity towards *N. caninum* on milk production (<100 kg per lactation) was evidenced in herds from Costa Rica (Romero et al., 2005). Besides, Hobson et al. (2002) reported that the association between *N. caninum* serostatus and milk production in Ontario Holstein dairy cattle depended on the abortion status of the herd. On the contrary, in a study from Australia, seropositive cows appeared to produce more milk (0.4 kg/cow/day) than seronegative ones (Hall et al., 2005); similarly, in New Zealand, seropositive cows were higher producers, suggesting that the farmer could be inclined to keep aborting cows if

they were of higher genetic/production value (Pfeiffer et al., 2002). Interestingly, milk production losses of 1.6 kg/cow/day on farms categorized as highly positive if compared to negative and low-positive herds were demonstrated (Gonzales-Warleta et al., 2011). However, since milk production depends on many factors, i.e., the farm effect, as the sum of genetics, management, and environmental effects, it is difficult to assess if neosporosis could have a substantial effect on productive parameters (Romero et al., 2005).

Concerning milk qualitative parameters, no difference was revealed in fat and protein content between positive and negative herds. However, the univariate analysis evidenced a higher prevalence of *N. caninum* related to farms with somatic cell counts > 300000. Similarly, Ould-Amrouche et al. (1999) reported that somatic cell counts between 200000 and 400000 were significantly related to *N. caninum* infection in cattle from France. However, a recent study from Iran observed no significant difference between the infection rate with *T. gondii* and *N. caninum* and the number of somatic cells (Gharekhami et al., 2021); besides, the study by Peregrine et al. (2004) conducted in Ontario dairy herds specifically investigating the potential association of *N. caninum* infection with udder health evidenced that seropositive cows had fewer intramammary environmental infections. Therefore, since somatic cell count is a parameter to evaluate milk quality, indicating an inflammatory response in the mammary gland, the association with *N. caninum* infection is still unclear.

The seropositivity to *N. caninum* of the herds from this study revealed a different distribution according to sampling administrative districts. Indeed, the provinces of Bergamo, Brescia, Cremona, and Milano had the highest prevalence values due to the large number of seropositive herds in the area, as confirmed by the SatScan significant central cluster of positive herds and the high probability area of infection risk evidenced by MaxEnt model, both falling within the territory of these provinces. It was observed that a few bioclimatic (temperature and precipitation) and environmental (altitude and use categories of agricultural and forest soil) variables play an important role in increasing the probability of occurrence of *N. caninum* positive herds. Several

studies demonstrated that temperature and precipitation could have an influence on oocysts sporulation and resistance in the environment (Schaes et al., 2003; Lopez-Gatius et al., 2005; Rinaldi et al., 2005; Dubey et al., 2007; Nogareda et al., 2013; Gazzonis et al., 2020). Particularly, Dubey (1999) determined that *N. caninum* oocysts sporulate within 24 h under optimal conditions (at 37°C or room temperature). In the study area, average temperatures are low in winter and high in summer; specifically, in summer, the maximum temperatures can reach, in the case of a subtropical anticyclone, peaks of 38°C or even higher values. Furthermore, the suitability of the area of the significant positive cluster for *N. caninum* is also determined by the specific geographical position of the Po Plain, which is open only on the eastern side and closed between high mountain chains that partially hinder the winds and favor the accumulation of strong humidity in the air.

The evidence that land use/land cover use variable had an effect on the positive cluster may suggest that the high number of positive herds inside the cluster could also be attributable to intensive farming and agriculture, but also to high anthropization and presence of dogs in the area. Previous studies have indicated, in addition to climatic characteristics, also the influence of demographic ones on the risk of protozoan infection in cattle: indeed, it was evidenced that dog density, and hence urbanization, are related to the parasite infection (Schaes et al., 2003; Frössling et al., 2008; Nasir et al., 2012). Then, the high interaction between dogs and cattle that probably occurs in the cluster area is of crucial importance for the biological cycle of *N. caninum* since, as already demonstrated, the vertical transmission alone is not sufficient to sustain protozoan infection (Bartels et al., 2006). On the other hand, the hypothesis that the study area is characterized by a high environmental contamination of oocysts is convincing and, as above reported, it could be supported by previous studies that in the same area showed the role of other animals as intermediate hosts and potential reservoirs of the parasite, including both domestic and wild species (Gazzonis et al., 2021; Villa et al., 2022a; 2023). Finally, it should also be considered that density of farms and herd consistency are higher inside than outside the central cluster area.

5. Conclusions

This study confirms a wide distribution of neosporosis in Italian dairy herds. Bulk tank milk serology is a useful tool to estimate and monitor *N. caninum* herd prevalence. The impact of the parasite on herd performances could be hypothesized, i.e. an effect in early pregnancy and on the productive parameters. On the basis of the SatScan model, *N. caninum* has been detected or is likely to occur in the central sector of the Po Plain, also in accordance with the environmental requirements identified in the MaxEnt model, characterized as warm and wet highly anthropized lowland areas, used for intensive farming and agriculture. Future climate projections models performed by Baronetti et al. (2022) showed an increase in minimum and maximum temperature for northern Italy, with a more complex behavior of precipitation anomalies. Indeed, for the near future (2021–2050), the Po Plain will be affected by global warming with an increase of annual maximum temperature of 2 °C. This scenario could affect parasite distribution along an altitudinal gradient and more suitable microclimatic conditions on sporulation and/or survival of oocysts, considering higher temperature and humidity as risk factors on seropositivity in herds (Dubey et al., 2007; Mendoza Morales et al., 2022). Therefore, it is underlined the importance of information on *N. caninum* and neosporosis for veterinarians and farmers who should be aware of neosporosis. Indeed, in-depth analyses should be conducted in seropositive herds, as well as for the adoption of control plans.

CRedit authorship contribution statement

Luca Villa: Conceptualization, Methodology, Investigation, Writing – original draft. Carolina Allievi: Methodology, Investigation, Data curation. Anna Rita Di Cerbo: Methodology, Software, Review. Sergio Aurelio Zanzani: Writing – review & editing. Flavio Sommariva: Investigation. Lucio Zanini: Investigation. Michele Mortarino: Conceptualization, Writing – review & editing.

Maria Teresa Manfredi: Conceptualization, Supervision, Resources, Funding acquisition, Writing – review & editing

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Tables

Table 1. List of WorldClim bioclimatic and environmental variables used in the Bernoulli model.

Variable	Description	Source
BIO01	Annual Mean Temperature	WorldClim https://www.worldclim.org/data/index.html
BIO02	Mean Diurnal Range (Mean of Monthly (max temp - min temp))	WorldClim https://www.worldclim.org/data/index.html
BIO03	Isothermality (BIO2/BIO7) ($\times 100$)	WorldClim https://www.worldclim.org/data/index.html
BIO04	Temperature Seasonality (Standard Deviation $\times 100$)	WorldClim https://www.worldclim.org/data/index.html
BIO05	Max Temperature of Warmest Month	WorldClim https://www.worldclim.org/data/index.html
BIO06	Min Temperature of Coldest Month	WorldClim https://www.worldclim.org/data/index.html
BIO07	Temperature Annual Range (BIO5-BIO6)	WorldClim https://www.worldclim.org/data/index.html

BIO08	Mean Temperature of Wettest Quarter	WorldClim https://www.worldclim.org/data/index.html
BIO09	Mean Temperature of Driest Quarter	WorldClim https://www.worldclim.org/data/index.html
BIO10	Mean Temperature of Warmest Quarter	WorldClim https://www.worldclim.org/data/index.html
BIO11	Mean temperature of Coldest Quarter	WorldClim https://www.worldclim.org/data/index.html
BIO12	Annual Precipitation	WorldClim https://www.worldclim.org/data/index.html
BIO13	Precipitation of Wettest Month	WorldClim https://www.worldclim.org/data/index.html
BIO14	Precipitation of Driest Month	WorldClim https://www.worldclim.org/data/index.html
BIO15	Precipitation Seasonality (Coefficient of Variation)	WorldClim https://www.worldclim.org/data/index.html
BIO16	Precipitation of Wettest Quarter	WorldClim

		https://www.worldclim.org/data/index.html
BIO17	Precipitation of Driest Quarter	WorldClim https://www.worldclim.org/data/index.html
BIO18	Precipitation of Warmest Quarter	WorldClim https://www.worldclim.org/data/index.html
BIO19	Precipitation of Coldest Quarter	WorldClim https://www.worldclim.org/data/index.html
DUSAF	Use Categories of Agricultural and Forest Soil	Geoportal of Lombardy https://www.geoportale.regione.lombardia.it/
WATER	Water Courses	Geoportal of Lombardy https://www.geoportale.regione.lombardia.it/
ELEV	Altitude	Geoportal of Lombardy https://www.geoportale.regione.lombardia.it/

Table 2. Prevalence values related to *Neospora caninum* infection in dairy cattle herds in Italy according to the considered herd data.

Variable	Category	N° Positive/Examined	Prevalence (95% CI)*
Herd province	Bergamo	40/96	41.7% (31.8-51.5)
	Brescia	40/99	40.4% (30.7-50.1)
	Cremona	33/86	38.4% (28.1-48.6)
	Lodi	12/89	13.5% (6.4-20.6)
	Mantova	16/66	24.2 % (13.9-34.6)
	Milano	32/99	32.3 % (23.1-41.5)
	Pavia	7/51	13.7% (4.3-23.2)
Herd consistency	Small	18/75	24.0% (14.3-33.7)
	Small-medium	98/245	40.0% (33.9-46.1)
	Medium	41/145	28.3% (20.9-35.6)
	Large	23/120	19.2% (12.1-26.2)
Herd age	≤ 2 years old	3/25	12.0% (-0.7-24.7)
	> 2 years old < 4	128/427	30.0% (25.6-34.3)
	≥ 4 years old	46/122	37.7% (29.1-46.3)
Mean number of inseminations for conception	< 2 inseminations	22/88	25.0% (16.0-34.0)
	2-3 inseminations	107/365	29.3% (24.6-34.0)
	> 3 inseminations	48/121	39.7% (31.0-48.4)
Period from calving to conception	≤ 150 days	50/238	21.0% (15.8-26.2)
	> 150 days	126/332	38.0% (32.7-43.2)
Daily milk yield	≤ 20 kg	49/122	40.2% (31.5-48.9)
	21-25 kg	64/170	37.6% (30.4-44.9)

	26-30 kg	31/141	22.0% (15.1-28.8)
	> 30 kg	20/98	20.4% (12.4-28.4)
305-mature equivalent milk yield	\leq 11000	132/349	37.8% (32.7-42.9)
	> 11000	35/156	22.4% (15.9-29.0)
Somatic cell counts	\leq 200000 cells	44/169	26.0 (19.4-32.7)
	> 200000 <300000 cells	86/281	30.6 (25.2-36.0)
	\geq 300000 cells	50/124	40.3 (31.7-49.0)

*95CI=95% confidence interval

Table 3. Results of the univariate analysis related to *Neospora caninum* infected dairy cattle herds in Italy.

Variable	Category	Odds ratio (95% CI*)	P value
Herd province	Bergamo	4.5 (1.8-11.0)	0.001
	Brescia	4.2 (1.7-10.4)	0.001
	Cremona	3.9 (1.6-9.7)	0.003
	Lodi	0.9 (0.3-2.7)	>0.05
	Mantova	2.0 (0.8-5.3)	>0.05
	Milano	3.0 (1.2-7.4)	0.017
	Pavia (PV)	1	
Herd consistency	Small	1.3 (0.7-2.7)	>0.05
	Small-medium	2.8 (1.7-4.7)	0.000
	Medium	1.7 (0.9-3.0)	>0.05
	Large	1	
Herd age	≤ 2 years old	1	
	> 2 years old < 4	3.1 (0.9-10.7)	>0.05
	≥ 4 years old	4.4 (1.2-15.6)	0.020
Mean number of inseminations for conception	< 2 inseminations	1	
	2-3 inseminations	1.2 (0.7-2.1)	>0.05
	> 3 inseminations	2.0 (1.1-3.6)	0.028
Period from calving to conception	≤ 150 days	1	
	>150 days	2.3 (1.6-3.4)	0.000
Daily milk yield	≤ 20 kg	0.4 (0.2-0.7)	0.002
	21-25 kg	0.4 (0.2-0.7)	0.003
	26-30 kg	0.9 (0.5-1.7)	>0.05

	> 30 kg	1	
305-mature equivalent milk yield	≤ 11000	2.1 (1.4-3.2)	0.001
	> 11000	1	
Somatic cell counts	≤200000 cells	1	
	>200000 <300000 cells	1.2 (0.8-1.9)	>0.05
	≥300000 cells	1.9 (1.2-3.1)	0.010
% Fat	Continuous variable	1.3 (0.7-2.2)	>0.05
% Protein	Continuous variable	0.7 (0.2-2.7)	>0.05

*95CI=95% confidence interval

Table 4. Results of the multivariate analysis related to *Neospora caninum* infected dairy cattle herds in Italy.

Variable	Category	Odds ratio (95% CI)	P value
Herd province	Bergamo (BG)	6.8 (2.1-22.0)	0.001
	Brescia (BS)	6.1 (1.9-19.3)	0.002
	Cremona (CR)	4.8 (1.5-15.5)	0.009
	Lodi (LO)	1.3 (0.3-5.1)	>0.05
	Mantova (MN)	2.5 (0.7-8.6)	>0.05
	Milano (MI)	3.3 (1.0-10.6)	0.046
	Pavia (PV)	1	
Herd consistency	Small	0.5 (0.2-1.2)	>0.05
	Small-medium	1.7 (1.0-3.2)	0.05
	Medium	1.4 (0.7-2.8)	>0.05
	Large	1	
Period from calving to conception	≤ 150 days	0.5 (0.3-0.8)	0.005
	>150 days	1	
305-mature equivalent milk yield	≤ 11000	2.2 (1.3-3.5)	0.001
	> 11000	1	

*95CI=95% confidence interval

Table 5. Significant clusters of *Neospora caninum* infected dairy cattle herds

Clusters	Coordinates	Herds	No of cases	Relative risk	<i>P</i> value
	Radius				
High risk cluster	45.518795 North-9.692540 East 20.99 km	155	78	2.13	<0.001
Low risk cluster	45.199158 North-9.576438 East 13.20 km	67	0	0	<0.001

Figure Legends

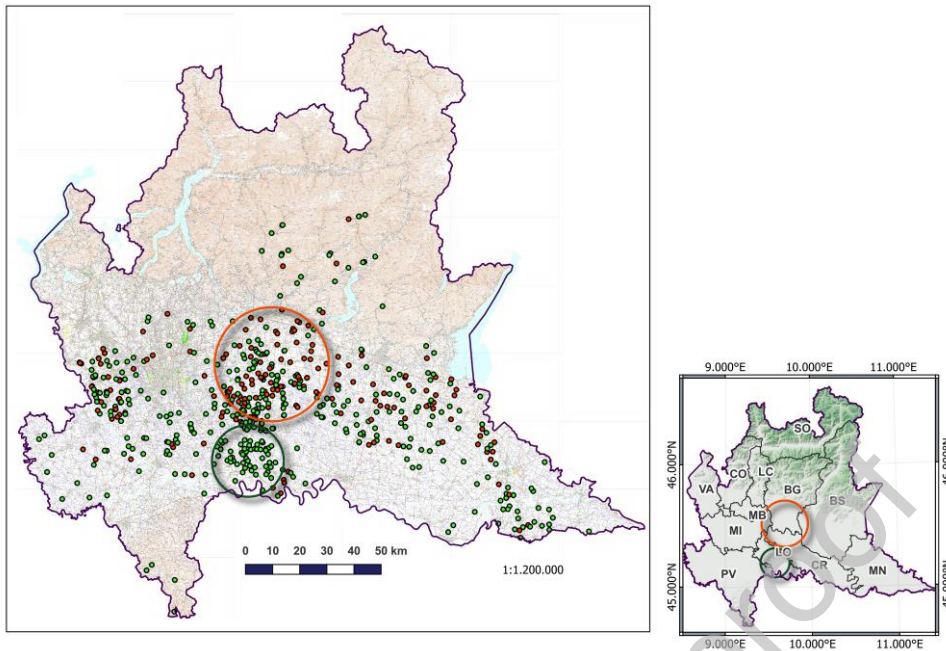


Figure 1. Bernoulli spatial SatScan analysis for *Neospora caninum* infection by bulk tank milk analysis from dairy cattle herds in Italy. Blue and red dots indicate negative and positive herds, respectively. The red circle indicates the central high-rate cluster and the blue circle indicates the southern low-rate cluster.

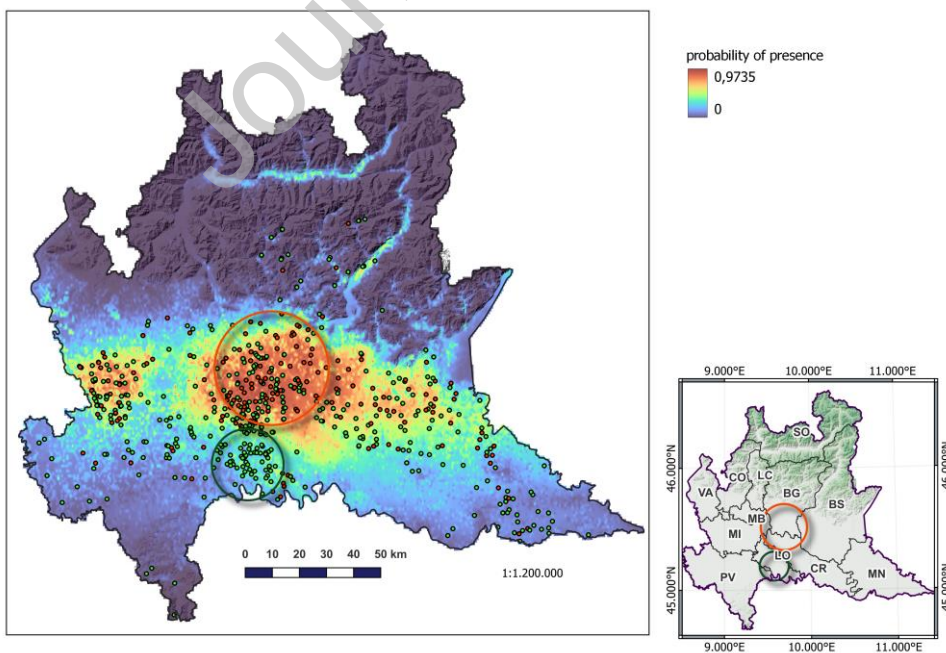


Figure 2. Maximum Entropy model analysis for *Neospora caninum* infection by bulk tank milk analysis from dairy cattle herds in northern Italy. The probability of *N. caninum* infection is represented by the color legend. Green and red dots indicate negative and positive herds, respectively. The red circle indicates the central high-rate cluster and the blue circle indicates the southern low-rate cluster evidenced by the Bernoulli spatial SatScan analysis.

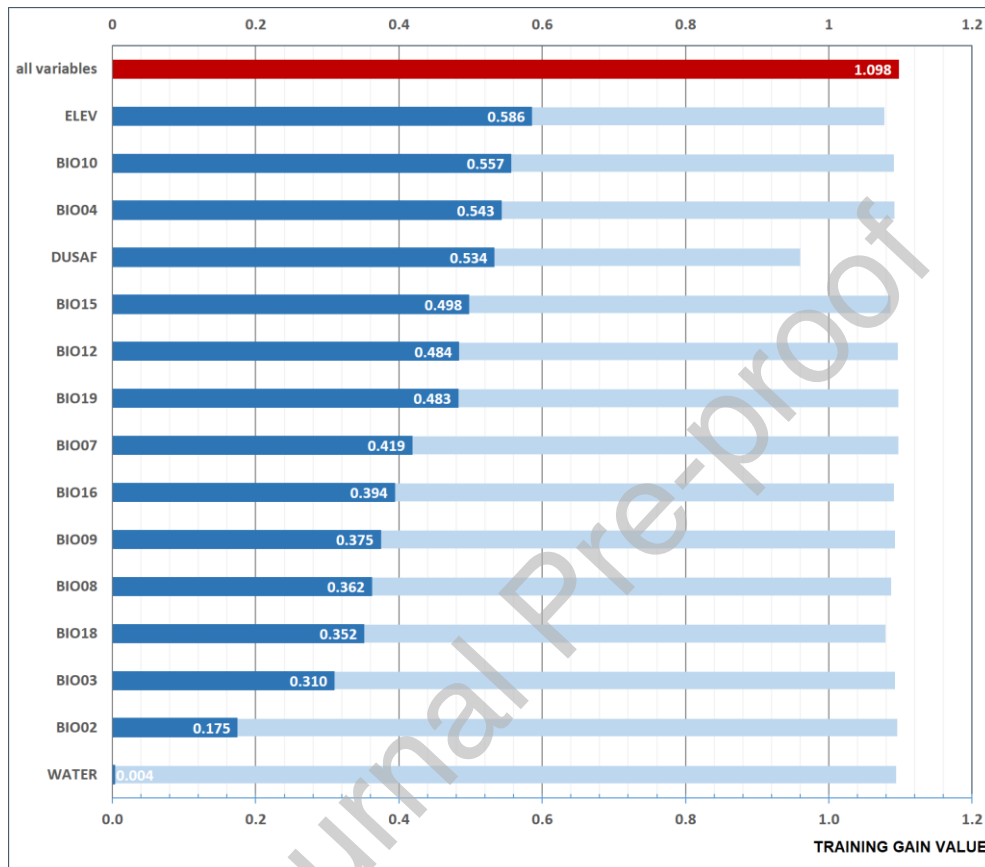


Figure 3. Jackknife test of individual bioclimatic and environmental variable importance in the development of the MaxEnt model relative to all variables (red bar) for each predictor variable alone (blue bars), and the drop in training gain when the variable is removed from the full model (light blue bars). Length of bar represents the training gain value. A longer bar represents a higher training gain.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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