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RESEARCH ARTICLE

Stability and purity of selected ryegrass *Epichloë* endophytes in New Zealand dairy pastures

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Abstract

Background: Perennial ryegrass (*Lolium perenne*) in New Zealand pastures is typically infected with the mutualist *Epichloë* fungal endophyte. This endophyte assists the plant in resisting biotic and abiotic stresses, but the standard strain of endophyte is toxic to livestock. Elite ryegrasses with selected endophytes have been developed to provide protective properties to the grass plant and lessen or eliminate the negative impacts on livestock.

Methods: Using immunology and molecular techniques, the presence of endophyte infection and endophyte strain in ryegrass tillers was determined for 24 dairy pastures sampled for up to 7 years in regions of the North and South Islands.

Results: In general, infection levels were high and showed small increases over time. Some pastures failed to reach 70% infection. The sown, selected endophytes were the dominant endophyte strains present and these were stable over time. Standard endophyte was the primary nonsown endophyte, and while generally low and so of little importance, it increased over time and for some pastures, this would have been detrimental to livestock. Pasture establishment technique influenced the level of contamination.

Conclusions: Results reinforce the importance of following best practice procedures in the seed industry and on-farm. Researchers should monitor trials for contaminating nonsown standard endophyte.

KEYWORDS

competition, *Epichloë*, *Lolium perenne*, pasture renewal, selected endophytes, toxicity

INTRODUCTION

Cultivated species of ryegrass (*Lolium perenne* L., *Lolium multiflorum* L., and their hybrid *Lolium* × *boucheanum* Kunth.) are grown worldwide, as they are well adapted to a wide range of temperate climates with moist, fertile soils (Jung et al., 1996). Fast establishment, high palatability, high yields of quality herbage, and tolerance to a range of grazing managements make it a pasture species of choice for many farmers worldwide. Ryegrasses are the most commonly sown species in New Zealand pastures, particularly on dairy farms, and are the most economically valuable plant species to the New Zealand economy (Nixon, 2016).

Ryegrasses, along with other species, were first introduced to New Zealand for use in pastures during the early migrations of Europeans in the 1800s (Caradus, Goldson, et al., 2021). Over time, it became apparent that local ecotypes from permanent pastures in New Zealand were agronomically superior to seed lines imported from the United Kingdom and Europe. This led to local development of cultivars by Government breeders in the 1930s and to the New Zealand certified seed production system (Stewart, 2006).

Unbeknown at the time, a key part of the success of locally adapted cultivars was most likely due to the insect-protective properties of high levels of the asexual, obligate, mutualistic, seed-transmitted *Epichloë* fungal endophyte infecting the perennial ryegrass (*L. perenne*) ecotypes (Caradus et al., 2023; Stewart, 2006; Stewart, Goldson, et al., 2022). A similar paradigm is likely with regard to the development of superior locally adapted endophyte-infected perennial ryegrasses in Australia and

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tall fescues (*Festuca arundinacea* Schreb. syn. *Lolium arundinaceum* [Schreb.] Darbysh.) in the United States (Ball et al., 2019; Reed, 2014).

In pivotal studies in the 1970s and the 1980s in New Zealand and the United States, the presence of asexual *Epichloë* endophytes was linked to superior grass performance along with detrimental effects on the health and productivity of a wide range of farmed ruminants and monogastrics, for both perennial ryegrass and tall fescue (Bacon et al., 1977; Fletcher & Harvey, 1981; Mortimer et al., 1982; Read & Camp, 1986). In further developments, researchers were able to attribute plant-protection and animal health effects to the presence or absence of particular alkaloids in the endophyte–grass associations. This led to commercial release of selected (/novel) endophyte strains for several closely related species of ryegrass (*L. perenne*, *L. boucheanum*, and *L. multiflorum*) and tall fescue (Caradus, Chapman, et al., 2021; Johnson et al., 2013; Stewart, Kerr, et al., 2022). These selected endophytes provided the host grass with protection against a range of biotic and abiotic stresses, but with few or none of the negative effects on livestock as occurred with the standard endophyte strain infecting the locally developed ecotype-based cultivars.

For ryegrasses in New Zealand, there has been rapid incorporation of selected endophytes into local cultivars, and high uptake by farmers. It is estimated that elite cultivars infected with selected strains of *Epichloë* endophyte are now 90% of the New Zealand proprietary perennial ryegrass seed market (Caradus, Chapman, et al., 2021). These selected endophytes have largely displaced the use of standard endophyte (also referred to as wild-type or common-toxic endophyte), which is a mammalian-toxic strain in sown ryegrasses. The standard endophyte strain is known to cause significant animal health issues and financial losses to livestock farmers (Hume et al., 2016).

To maximize the animal productivity and welfare benefits of selected endophytes, elite cultivars with selected endophyte need to be competitive with the widely naturalized standard endophyte-infected ryegrass. The naturalized ryegrass may reinvade pastures via seed or incomplete kill of the old pasture (Hume & Barker, 2005). In New Zealand, under experimental small plot conditions, standard endophyte-infected ryegrass has been proven to be highly competitive with ryegrass infected with the AR1 selected endophyte strain when sown in a 50–50 seed mix (Hume & Barker, 2005).

While there is a good understanding of the science and practices required to develop and deliver on-farm ryegrass seed that is highly infected with a selected endophyte (Johnson & Caradus, 2019), little has been measured and reported of endophyte infection for pastures grazed in commercial settings. To address this knowledge gap, in this study, we utilized dairy pastures in the North Island and South Island, most of which were part of a project investigating the value of pasture renewal and drivers of pasture persistence. Full details of the renewal–persistence project, along with the initial endophyte results, have been described by Rennie et al. (2011), Taylor et al. (2012), and Tozer et al. (2015). Here, we report on the change over time in the percentage of tillers infected with endophyte and the endophyte strain in ryegrass sampled for up to 7 years from newly (<1 year) or recently (2–6 years old) renovated pastures. This expands on a preliminary conference publication of these results by Hume et al. (2023).

MATERIALS AND METHODS

Sites sampled

The ryegrass (*L. perenne*) pastures sampled in the upper North Island, from the renewal–persistence project, were from the Waikato and eastern Bay of Plenty regions (37.6°–38.2° S) (Waikato-BoP). Across nine farms, a total of 13 pastures sown from 2005 to 2010 were monitored for a further 6 years beyond that reported by Rennie et al. (2011). By the final sampling, pastures ranged from 6 to 11 years of age. The 13 pastures were sown in one of six cultivar–endophyte combinations and three unknown cultivars, with Bealey NEA2 being the most common (Table 1). Five pastures had been sown into fully cultivated seed beds and eight direct-drill sown (no-till seed bed) into pasture that had been killed with herbicides (Table 1).

TABLE 1 Ryegrass cultivars infected with selected *Epichloë* endophytes sown in 13 upper North Island pastures (Waikato-BoP) and 10 South Island pastures, either into fully cultivated or herbicide-treated no-till seed beds.

Cultivar (ploidy)	Endophyte	Seed bed	Number of pastures
North Island (Waikato-BoP)			
Alto (D)	AR37[a]	Cultivated	1
Arrow (D)	AR1[b]	No-till	1
Bealey (T)	NEA2[b, c]	Cultivated	2
		No-till	2
Bealey (T) & Arrow (D) mix	NEA2/AR1	No-till	2
Grasslands Commando (D)	AR37	Cultivated	1
Extreme (D)	AR37	No-till	1
Unknown (T)	AR5[b]	No-till	1
Unknown cultivars (D)	AR1	Cultivated	1
		No-till	1
South Island			
Halo (T)	AR37	Cultivated	3
		No-till	4
Samson (D)	AR37	Cultivated	2
		No-till	1

Abbreviations: BoP, Bay of Plenty; D, diploid; T, tetraploid.

[a]*Epichloë* species LpTG-3.

[b]*Epichloë festucae* var. *lolii*.

[c]Marketed as "NEA2"—a blend of nea2 and nea6 strains of endophyte (Eady, 2021).

An additional, irrigated ryegrass pasture was sampled in the eastern Bay of Plenty (BoP), in the Te Teko District (38.0° S), which was not part of the Waikato-BoP renewal–persistence project. This had been sown in 2010 with a seed lot of Bealey NEA2, which unknowingly had a low proportion of viable endophyte (pasture was 37% endophyte-infected as tested 6 months after sowing [unpublished data]). This pasture had been sown into a fully cultivated seed bed, with the previous pasture having been killed with herbicides.

The ryegrass (*L. perenne*) pastures sampled in the South Island from the renewal–persistence project were from irrigated areas of the eastern coastal region, extending from North Canterbury to North Otago (42.8°–45.0° S) (Taylor et al., 2012). Ten pastures, each from a different farm, were sown in either autumn ($n = 8$) or spring ($n = 2$) of 2010. Two AR37-infected cultivars were sown equally into either a fully cultivated seed bed or a no-till seed bed for pasture that had been killed with herbicides (Table 1). One of the cultivated pastures was on an organic farm, with no use of herbicides to kill the old pasture.

Tiller sampling

At each sampling date, vegetative ryegrass tillers were selected from the pasture at regular intervals along a diagonal transect of the length of each paddock, with each tiller taken from a different plant. Tillers were cut at ground level with a knife or scalpel. Once paddock sampling was complete, all tillers were sealed in a plastic bag and kept cool (~10°C) while being transported to the laboratory, where they were stored at 4°C until processing for endophyte infection.

The 13 North Island Waikato-BoP pastures were sampled annually for 7 years (2009–2015) in late spring or early summer for determination of endophyte presence (100 tillers per pasture per sampling time), and from these tillers, subsets of 40 tillers per pasture per sampling time were tested for endophyte strain.

The Te Teko North Island pasture of Bealey NEA2 (a blend of nea2/nea6 strains) was tested in autumn for 7 years (2014–2019, 2021; 4, 5, 6, 7, 8, 9, and 11 years after sowing) for endophyte presence (mean of 228 tillers per year) and for 5 years (5, 7, 8, 9, and 11 years after sowing) for endophyte strain (mean of 76 tillers per year). For this pasture, only tillers that tested positive for endophyte presence were then tested for endophyte strain.

The 10 pastures in the South Island were sampled annually in late summer/mid-autumn for 7 years (2010–2016) for endophyte presence (50 tillers per pasture) and endophyte strain (40 tillers per pasture in each of 2012 and 2013, and 50 tillers in 2016).

Endophyte detection and strain identification

The presence of endophyte in tillers was determined using a tissue print immunoblot assay, and then *Epichloë* endophyte strain was determined using DNA simple sequence repeat (SSR) markers.

Within 24 h of sampling, any dead matter was removed from the base of tillers in preparation for the immunoblot assay. A scalpel was used to cut each tiller transversely ~2 mm from the base. The freshly cut end was then gently pressed onto a 0.45 µm nitrocellulose membrane to transfer any endophyte proteins to the membrane. Once all tillers had been processed, the membranes were subjected to a series of development steps involving a milk protein blocking solution (Tris(hydroxymethyl) methylamine, NaCl, nonfat milk powder, 1M HCl, RO water), a primary antibody (rabbit antiendophyte), then a secondary antibody (goat anti-rabbit) and finally a chromogen to visualize endophyte-infected and endophyte-free tillers (Simpson et al., 2012). From these data, the frequency of endophyte infection was calculated and presented as a percentage of infected tillers.

The SSR testing was undertaken by extracting DNA from the basal 20 mm of each tiller using the method of Anderson et al. (2018). DNA extracts were typed for endophyte strain using the SSR markers B11 (Moon et al., 1999), ans025, and ans031 (Card et al., 2014). This combination of markers enabled the detection of standard endophyte and known commercial endophyte strains AR1, AR5, AR37, nea2, and nea6. Polymerase chain reaction (PCR) for B11 was performed as described by Moon et al. (1999), except that a 10 µL reaction volume was used, and for ans025 and ans031, PCR was conducted as described in Sartie et al. (2011). For all SSR markers, capillary electrophoresis of PCR products was performed using an ABI 3130xl genetic analyzer (Applied Biosystems) as described in Sartie et al. (2011). Electropherograms were analyzed and fragments were sized using GeneMarker v1.75 software (SoftGenetics, LLC). Using these data, the frequency of differing endophyte strains was calculated and presented as a percentage of infected tillers.

Statistical analysis

The immunoblot data assessing endophyte presence/absence were subjected to distinct analyses for (1) Waikato-BoP North Island pastures, (2) Te Teko North Island pasture, and (3) South Island pastures.

For the Waikato-BoP North Island pastures, a pairwise proportion test was used to examine differences in response proportion across various years. A semiparametric regression with a binomial distribution was applied to model the response with a nonlinear trend over the years, while a logistic regression captured the response with a linear trend. The model incorporated fixed effects for Year, Seed Bed, and their interaction up to two levels. A random effect for Paddock, nested within Farm, was included. As pastures were sown over a 5-year period, a covariate of Years Since Sowing was tested.

For the Te Teko North Island pasture and the South Island pastures, a logistic mixed model was utilized. For the South Island pastures, this model featured fixed effects such as Year, Group (High, Moderate), Seed Bed (Cultivated, No-till), and interactions up to two levels.

The modeling results were interpreted through predicted means, with multiple comparisons conducted and *p*-values adjusted using the "BH" method for significant effects. All statistical analyses were executed in R 4.3.2, utilizing the "lme4" and "predictmeans" packages (Luo et al., 2021). Significance was determined at the 0.05 level. Confidence intervals (CIs) for means are presented at the 84.3% level so that nonoverlapping CIs for a pair of treatments correspond to a significant difference (at the 0.05 level) in those treatment means (Payton et al., 2003).

RESULTS

North Island Waikato-BoP pastures

Endophyte-infected tillers

The 13 Waikato-BoP North Island pastures had a mean of 89% endophyte-infected tillers over the 7 years of sampling (Table 2). On average, all 13 pastures had more than 70% endophyte-infected tillers (range 73%–96%; data not presented). There were differences between years (nonlinear response, $p < 0.001$), and the covariate of Years Since Sowing was significant ($p < 0.001$). There was no difference between the first (89%) and last year (88%) of measurement based on confidence intervals ($p > 0.05$) or pairwise comparisons ($p = 0.360$).

Nonsown endophytes

The overall mean of nonsown endophytes was 8% of the total tillers tested (Table 2). Nonsown endophytes were detected in three quarters of the samples (38 out of 51) taken over the 4 years, and were detected in all 13 pastures in one or more years. Of the 268 nonsown endophyte-infected tillers detected, most were standard endophyte (252), with low numbers of AR1 (7) and AR37 (9).

The nonsown endophytes occurred primarily in three pastures (182 tillers; mean of 37% of total tillers tested), and were almost all standard endophyte (179) (Table 3). In comparison, nonsown endophytes in the other 10 pastures were low (86 tillers; mean of 5% of total tillers tested) and mainly standard endophyte (73) (Table 3). Over time, endophyte increased in the high pasture group but not in the low pasture group (interaction $p = 0.029$).

Seed bed preparation

Seed bed preparation affected total endophyte-infected tillers (mean effect $p < 0.001$), but there was an interaction with year ($p = 0.004$). In this interaction, infection in pastures sown into cultivated seed beds increased over time (predicted means: increasing from 55% to 94%), while infection in the pastures sown into no-till seed beds showed a decline over time (predicted means: decreasing from 98% to 79%).

Contamination with standard endophyte was less in cultivated (predicted mean 13%) than no-till seed beds (predicted mean 26%) ($p = 0.043$).

TABLE 3 Predicted means of ryegrass tillers infected with nonsown *Epichloë* endophytes for the Waikato-BoP North Island pastures grouped into pastures with high ($n = 3$) and low ($n = 10$) levels of nonsown endophytes, expressed as a percentage of total tillers tested.

Grouping	Year of sampling			
	2010	2012	2013	2014
	Nonsown endophytes (%)[a]			
High-infected	19	36	40	51
Confidence interval (83.4%)	5–48	18–60	21–63	27–75
Low-infected	2	4	8	4
Confidence interval (83.4%)	1–7	2–7	4–13	2–9

[a]Determined by simple sequence repeat analysis.

TABLE 2 Predicted means of ryegrass tillers infected with *Epichloë* endophyte for the 13 Waikato-BoP North Island pastures as tested for endophyte presence only, and a subset of tillers tested for endophyte strain, all expressed as a percentage of total tillers tested.

Measurement	Year of sampling						
	2009	2010	2011	2012	2013	2014	2015
	Endophyte presence (%)[a]						
Total infected tillers	89	95	91	84	90	88	88
Confidence interval (83.4%)	70–97	89–98	84–95	77–90	84–93	79–94	74–95
	Endophyte strain (%)[b]						
Sown endophyte/s		80.3		74.5	58.4	76.0	
Standard endophyte (nonsown)		2.8		6.6	8.1	9.2	
Other endophytes (nonsown)		0.4		1.6	2.7	1.1	
All endophytes		83.5		82.7	69.2	86.3	

Abbreviation: BoP, Bay of Plenty.

[a]Determined by immunoblotting; does not distinguish between *Epichloë* strains.

[b]Determined by simple sequence repeat analysis.

North Island Te Teko pasture

In the Te Teko North Island pasture sown with Bealey NEA2, the proportion of endophyte-infected tillers increased over the 7-year period of measurement in a nonlinear manner ($p < 0.001$) (Table 4). There was a significant difference between the first (24%) and last year (52%) of measurement based on confidence intervals ($p < 0.05$) and pairwise comparisons ($p < 0.001$). Over this period of measurement, the mean increase in infected tillers was four percentage units per annum.

Of the nonsown endophyte-infected tillers, 411 tillers were infected with standard endophyte and 1 was infected with AR37. The overall mean of standard endophyte was 14% of the total tillers tested (Table 4). The response over time was close to significance ($p = 0.055$); the first (6%) and last (23%) years of measurement were different based on confidence intervals ($p < 0.05$) and pairwise comparisons ($p < 0.001$).

North Island pastures sown with mixtures of endophyte

The relative proportion of endophyte strain nea2 to nea6 (overall mean ratio 85:15) in cultivar Bealey NEA2 did not change over time in the six Waikato-BoP North Island pastures ($p = 0.239$) and the one Te Teko North Island pasture ($p = 0.200$). Seed Bed ($p = 0.439$) and Years Since Sowing ($p = 0.981$) had no effect on this ratio for the Waikato-BoP North Island pastures.

For the two Waikato-BoP North Island pastures sown with the Bealey NEA2/Arrow AR1 mix, AR1 was less than 1% of tillers in one pasture and averaged 14% (range 10%–18%) in the other pasture, with no change over time ($p = 0.792$).

South Island pastures

Endophyte-infected tillers

On average, 80% of ryegrass tillers were endophyte-infected in the 10 South Island pastures over the 7 years of sampling (Table 5). Over this period, there was a small linear increase ($p < 0.001$) in endophyte-infected tillers, with a trend, but no statistically confirmed difference between the first (77%) and last (83%) years of measurements based on confidence intervals ($p > 0.05$) and pairwise comparisons ($p = 0.055$). Overall, there was an average increase of one percentage unit per annum.

On analysis, the 10 pastures were found to belong to one of two groups: either a group of five highly infected (mean 87%) or a group of five moderately infected pastures (mean 69%) ($p < 0.001$). Three of the pastures in the moderately infected group were less than 70% infected (means 61%, 65%, 66%). One of these pastures was from the organic farm, where no herbicides were used to kill the pasture before cultivating the seed bed. Both groups showed similar changes over time (interaction $p = 0.371$).

Nonsown endophytes

In contrast to the North Island, frequency of nonsown endophytes was low in South Island pastures (predicted mean 4% of the total tillers tested) (Table 5). These nonsown endophytes were detected in 7 of the 10 pastures, and in approximately only half the samples (14 out of 26) taken over the 3 years. Of the 48 tillers with nonsown endophytes, 24 were standard endophyte, 18 AR1, and six could not be assigned to a known endophyte strain.

For the seven pastures where contamination was detected, predicted mean contamination was typically ≤7%, except for one pasture, which averaged 20% of total tillers tested. In this case, the contaminating endophyte was predominantly AR1 (16 out of 18 tillers), in a pasture that was known to have only partial herbicide kill during the pasture renewal process (Taylor et al., 2012).

The content of total nonsown endophyte was relatively constant in the moderately infected pasture group but increased with time in the highly infected pasture group (year × group interaction, $p = 0.018$).

Seed bed preparation

For the frequency of infected tillers in the South Island pastures, seed bed preparation had no main effect ($p = 0.581$) and no interactions ($p \geq 0.094$).

TABLE 4 Predicted means of ryegrass tillers infected with *Epichloë* endophyte for the Te Teko North Island pasture as tested for total endophyte presence and presence of only standard endophyte, all expressed as a percentage of total tillers tested.

	Year of sampling							
Measurement	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**	**2021**
Total infected tillers (%)[a]	24	33	44	36	55	42	40	52
Confidence interval (83.4%)	21–27	30–36	42–47	33–39	52–58	39–45	32–47	49–56
Standard endophyte (nonsown) (%)[b]		6	8	12	20	14	14	23
Confidence interval (83.4%)		5–8	6–10	10–14	18–22	12–16	11–17	20–26

Note: Pasture was sown in 2010 with cultivar Bealey NEA2.

[a]Determined by immunoblotting; does not distinguish between *Epichloë* strains.

[b]Determined by simple sequence repeat analysis.

TABLE 5 Predicted means of ryegrass tillers infected with *Epichloë* endophyte for the eastern South Island as tested for endophyte presence only, and a subset of tillers tested for endophyte strain, all expressed as a percentage of total tillers tested.

Measurement	Year of sampling						
	2010	2011	2012	2013	2014	2015	2016
	Endophyte presence (%)[a]						
All pastures	77[b]	78	79	80	81	82	83
Confidence interval (83.4%)	71–82	72–83	73–84	75–84	76–85	77–86	78–87
	Endophyte strain (%)[b]						
Sown AR37			82.7	73.9			81.1
Nonsown endophytes			2.4	3.5			6.3
All endophytes			85.1	77.4			87.4

Note: Mean of 10 pastures sown in 2010 with AR37-infected cultivars.

[a]Determined by immunoblotting; does not distinguish between *Epichloë* strains.

[b]Determined by simple sequence repeat analysis.

All pastures established by no-till were contaminated, with approximately half the nonsown endophytes being standard and half AR1. This included the pasture with only partial herbicide kill of the previous AR1–ryegrass pasture. For the pastures sown into cultivated seed beds, nonsown endophytes were only present in two of the five pastures, and all were the standard endophyte. Seed bed preparation did affect ($p < 0.001$) total nonsown endophytes, with predicted means of 5.5% and 0.5% for no-till and cultivated seed beds, respectively. A similar effect was apparent for the nonsown standard endophyte ($p = 0.016$).

DISCUSSION

Endophyte tiller infection

Of the 24 pastures monitored in this study, most had high levels of endophyte-infected ryegrass tillers (>70%). This was particularly so for the group of 13 pastures in the upper North Island (Waikato-BoP), with a mean of 89% infection. Four pastures had less than 70% infection, the most notable being the Te Teko North Island pasture at 41%. The cause of the <70% infection in one of these South Island pastures is likely a result of the pasture renewal process failing to kill all the endophyte-free plants from the old pasture as no chemical control was used on this organic farm (Taylor et al., 2012).

The proprietary forage seed industry has agreed that seed should not be sold as "infected with a viable *Epichloë* endophyte" if less than 70% infected, as otherwise, it will be less efficacious in the field (Johnson & Caradus, 2019). The results from the 24 commercial pastures suggest that the industry has largely been successfully delivering and establishing ryegrasses infected with selected endophytes on dairy farms, and endophytes have maintained a high frequency in the pastures over time. To achieve this, there are varying interdependent steps that influence endophyte infection, including the processes of cultivar–endophyte development, seed production, seed storage, seed distribution, and the pasture establishment process (Hume & Barker, 2005; Johnson & Caradus, 2019). Apart from the organic farm, our study could not identify specific points in this chain that could explain the pastures in this study with <70% endophyte-infected tillers.

Change over time in infection levels

There were small increases in the proportion of endophyte-infected tillers over time in two of the pasture groupings analyzed (South Island, Te Teko North Island) and mixed evidence for an increase in the 13 Waikato-BoP North Island pastures. The magnitude of the increase was greatest for the Te Teko North Island pasture at four percentage units increase per annum. This pasture had the lowest initial value of 24% infected tillers. The 10 South Island farms increased at one percentage unit per annum, with similar increases in both the highly and moderately infected groups. For the 13 Waikato-BoP North Island pastures, the regression analysis showed significant differences between years. This appeared to reflect fluctuations between years (range 84%–95%), but inclusion of Years Since Sowing in the model indicated that older pastures had higher frequencies of infected tillers. Annual increases of ~1 percentage point per annum are unlikely to be of importance for perennial ryegrass pastures under dairy grazing in New Zealand, where annual pasture renewal rates are relatively high, typically 6%–8% (Kerr et al., 2015).

Changes in the proportion of endophyte-infected ryegrass tillers has been documented for numerous New Zealand ryegrass studies since the 1980s (Hume & Barker, 2005). In most but not all cases, endophyte-infected tiller infection increased over time. Similar results are reported for ryegrass in Australia and tall fescue in the United States (Hume & Barker, 2005; Hume & Sewell, 2014). In some cases in New Zealand, the increases have been considerably greater than the 1%–4% per annum found in the current study. For example, increases of over 20 percentage units per annum were reported by Francis and Baird (1989) in Canterbury and by Thom et al. (1999) in the Waikato. There is

evidence that increases may be less (or values stable) where infections levels are low or high, but this is not universal (Hume & Barker, 2005). The results presented in the current study provide some support for this.

The increases over time in the proportion of endophyte-infected tillers indicate that biotic and/or abiotic selection pressures were favoring endophyte-infected tillers over endophyte-free tillers, as expected. Invertebrate pests are known to be a major selective force in endophytic grass pastures in New Zealand (Caradus, 2023), which can be compounded by drought events (Hewitt et al., 2021), and why selected endophytes are rigorously tested to determine the degree of insect pest control (Caradus, Chapman, et al., 2021). Pest pressure will vary between regions, within regions, soil types, and years. The degree of change, or lack of it, is indicative of the degree of stress that endophytes can alleviate. Irrigation on all the South Island pastures, and the Te Teko North Island pasture, is likely to have mitigated some of the insect pressure, if present.

Contamination

Overall, the presence of contaminating (nonsown) endophytes was low, ranging from a mean of 4% for the South Island pastures to 8% in the Waikato-BoP North Island pastures, and 15% in the other North Island pasture at Te Teko. The majority (94% of nonsown tillers) of these endophytes were the standard endophyte strain, particularly in the North Island (98% of nonsown tillers). In the South Island, approximately half the contaminating endophytes were standard endophyte, with AR1 being the most abundant other nonsown strain present and primarily occurring in just one pasture.

There was clear evidence in the North Island for increasing contamination from nonsown standard endophyte over time. Although standard endophyte was present in all 14 North Island pastures, the increases over time were confined to just four pastures. While contamination was much lower and less frequent in the South Island, again, there was evidence for standard endophyte increasing over time but only in the highly infected pasture group.

Standard endophyte being the predominant nonsown endophyte, and increasing over time in some pastures, is not unexpected, as ryegrass infected with standard endophyte is widespread in the New Zealand landscape (Easton et al., 1996; Johnson et al., 2013). This was of no real concern in the South Island, where contamination was quite low (2% standard endophyte of total tillers tested). However, in three of the 13 Waikato-BoP North Island pastures, contamination increased substantially over time to be on average over half of the tillers in these pastures. At this level, standard endophyte would most likely be reducing the animal production and health benefits of the sown selected endophytes (Johnson et al., 2013). The other North Island pasture at Te Teko appeared to be on the same path, with increases in total endophyte infection being primarily due to the increase in standard endophyte.

Several previous studies in New Zealand have reported results of endophyte contamination testing, primarily for pastures grazed by dairy cattle. In general, contamination has been considered low and was primarily standard endophyte, and in some cases, increased over time (Faville et al., 2020; Hume & Barker, 2005; McNeill et al., 2007; Tozer et al., 2024; Ussher & Hume, 2015). As in the current study, there were a few pastures on commercial farms where standard endophyte contamination of the sown selected endophyte–ryegrasses was considerable, being in the range of 20%–50%.

The degree of competitiveness of the AR1 selected endophyte strain against standard endophyte has been evaluated in experimental small plot conditions at four sites for up to 4.5 years. In a 50:50 seed mix of AR1 and standard endophytes in the same cultivar, the standard endophyte-infected tillers increased in proportion to AR1-infected tillers at all sites, with a mean increase in standard endophyte of 10 percentage points per annum (Hume & Barker, 2005). There may be variation between selected endophyte strains in their competitive ability against standard endophyte, with Tozer et al. (2024) reporting greater standard endophyte contamination in nea2/6-infected cultivars than in equivalent AR37-infected cultivars, averaged over a 3-year study (6% vs. 3%, respectively).

The differential competitive ability of selected and standard endophytes may well be explained by their differing pest protection profiles as determined by differing endophyte alkaloid spectra (Caradus, Chapman, et al., 2021). For example, AR1-infected ryegrasses lack the mammalian toxic alkaloid ergovaline, an alkaloid that provides strong protection from adult African black beetles (*Heteronychus arator*). AR1-infected tillers therefore suffer more black beetle damage than standard endophyte-infected tillers (Caradus, Chapman, et al., 2021).

Sources of contamination

As reviewed by Hume and Barker (2005), pathways by which pastures can become contaminated with nonsown ryegrass endophytes are through (i) the incomplete kill of plants in the old pasture during the pasture renewal process and (ii) seed. The asexual *Epichloë* endophytes in pasture grasses do not transfer with grass pollen or by physical contact (Christensen & Voisey, 2007; Siegel, 1984). Contamination via seed can include contamination in sown seed, natural reseeding occurring before or after pasture renewal, transfer in dung by livestock, hay feeding on the pasture, and under- or over-sowing of ryegrass with a different endophyte by the farmer. Of these possible avenues of contamination, only the role of the renewal process for the 24 pastures monitored could be examined.

In general, cultivation is expected to be more effective than no-till (herbicide-treated and direct-drilled) in eliminating or at least reducing the carryover of endophyte-infected plants and or seed from the old pasture (Hume & Barker, 2005) and this will vary depending on the specifics of each pasture (Bluett et al., 2004; Burggraaf & Thom, 2000; Hume & Lyons, 1992). This was the case in the current study for mean levels of contamination for no-till and cultivated seed beds, particularly so for the South Island pastures.

Of the three North Island pastures that exceeded 50% contamination, two were no-till seeded and one sown into a cultivated bed. For the South Island pasture with the highest contamination (20%), the old pasture was only partially killed by the applied herbicide before no-till seeding (Taylor et al., 2012). In this case, AR1 was the main contaminating endophyte, an endophyte strain that would not compromise animal health or welfare, as AR1-infected ryegrass has none of the mammalian toxic alkaloids that are present in ryegrass infected with standard endophyte.

Ratio of endophytes nea2 to nea6

For the seven paddocks sown with cultivar Bealey NEA2, the relative proportion of endophyte strains nea2 (85%) to nea6 (15%) was the same as that reported to be sold in seed for sowing (Eady et al., 2017). These strains differ in the endophyte alkaloids that they express and the concentration of alkaloids. This is likely to favor nea6-infected tillers in terms of pest protection due to considerably higher ergovaline alkaloid concentrations than nea2-infected tillers (Popay et al., 2021). There was no evidence that this occurred for the seven paddocks monitored, as there was no change in this ratio over time.

This contrasts with the findings of Tozer et al. (2024) in a replicated small plot experiment under cattle grazing at Ruakura, Waikato. In this experiment, nea6 increased in proportion relative to nea2 over 3 years, as may have been predicted based on differing alkaloid profiles. These studies differed for ryegrass cultivar and ratios in the sown seed. The Ruakura experiment used three diploid cultivars, while the commercial paddocks that we monitored were all the tetraploid cultivar Bealey. At Ruakura, the sown seed was mixed in equal proportions of nea2 and nea6 (50:50), and not the 85:15 proportion in cultivar Bealey.

CONCLUSIONS

This study of commercial North and South Island dairy pastures has shown that in general, the industry has been successfully establishing ryegrass pastures with high levels of *Epichloë* endophyte-infected tillers, at least for the sowing period of 2005–2010. High levels of endophyte infection are a key component of productive, persistent ryegrass pastures under the biotic and abiotic stresses that can occur in the New Zealand environment (Caradus, 2023; Hume et al., 2020). Only a minority of the pastures were less than the desired 70% infection minimum, and in one case (organic farm), this is likely to have occurred as a result of decisions made in the pasture renewal process. This study also highlighted that the choice of pasture renewal method influenced the degree of contamination from standard endophyte, with cultivation resulting in less contamination than no-till. Standard endophyte is a strain that is widespread in New Zealand ryegrasses, is often competitive against selected strains, and its presence at moderate or high levels undermines the benefits of selected endophytes for animal production and health. Researchers need to be cognizant of this and monitor for contamination in research trials. Recommendations to farmers, and practices by the seed industry, need to reinforce the best management practices to reduce the likelihood of standard endophyte contamination.

AUTHOR CONTRIBUTIONS

David E. Hume: Conceptualization; data curation; funding acquisition; investigation; methodology; project administration; supervision; writing—original draft; writing—review and editing. **Dongwen Luo**: Formal analysis. **Grant M. Rennie**: Data curation; investigation; writing—review and editing. **Warren M. King**: Conceptualization; funding acquisition; investigation; methodology; project administration; supervision; writing—review and editing. **Anna L. Taylor**: Data curation; investigation; writing—review and editing. **Marty J. Faville**: Data curation; investigation; methodology; writing—review and editing. **Katherine N. Tozer**: Funding acquisition; investigation; project administration; supervision; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data are available on request.

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