Successful treatment of asymptomatic or clinically terminal bovine *Mycobacterium avium* subspecies *paratuberculosis* infection (Johne disease) with the bacterium Dietzia used as a probiotic alone or in combination with dexamethasone

Adaption to chronic human diarrheal diseases

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A naturally occurring gastrointestinal disease, primarily of ruminants (Johne disease), is a chronic debilitating disease that is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). MAP infection occurs primarily in utero and in newborns. Outside our Dietzia probiotic treatment, there are no preventive/curative therapies for bovine paratuberculosis. Interestingly, MAP is at the center of controversy as to its role in (cause of) Crohn disease (CD) and more recently, its role in diabetes, ulcerative colitis, and irritable bowel syndrome (IBS); the latter two, like CD, are considered to be a result of chronic intestinal inflammation. Treatments, both conventional and biologic agents, which induce and maintain remission are directed at curtailing processes that are an intricate part of inflammation. Most possess side effects of varying severity, lose therapeutic value, and more importantly, none routinely result in prevention and/or cures. Based on (a) similarities of Johne disease and Crohn disease, (b) a report that Dietzia inhibited growth of MAP under specific culture conditions, and (c) findings that Dietzia when used as a probiotic, (i) was therapeutic for adult bovine paratuberculosis, and (ii) prevented development of disease in MAP-infected calves, the goal of the present investigations was to design protocols that have applicability for IBD patients. Dietzia was found safe for cattle of all ages and for normal and immunodeficient mice. The results strongly warrant clinical evaluation as a probiotic, in combination with/without dexamethasone.

Introduction

Crohn disease (CD), ulcerative colitis (UC), and irritable bowel syndrome (IBS), sub-phenotypes of inflammatory bowel disease (IBD), are generally considered a result of chronic gastrointestinal inflammation. Based primarily on histopathology, genetic predisposition, and effective prophylactic treatments, this inflammation has been postulated to be a consequence of an unregulated immune response due to lack of regulator cell function, deletion of antigen-reactive cells (oral mucosal tolerance), loss of epithelial barrier architecture, and the most recent model, innate immune deficiency/dysfunction.\(^1\)\(^-\)\(^3\) Selection of an optimum treatment depends on several factors, including disease severity and location, co-morbidities, previous response to specific therapeutics, and the presence of surgical resection. Conventional treatments are directed at achieving symptomatic relief and preventing relapses via interference with immune processes. They include anti-inflammatory drugs (azathioprine, 6-mercaptopurine), steroids\(^4\)\(^-\)\(^5\) and more recently, biologic agents; infliximab,\(^6\)\(^-\)\(^7\) adalimumab,\(^8\)\(^-\)\(^9\) certolizumab pegol\(^10\) and natalizumab.\(^11\) Major drawbacks of the older modalities are: many fail over prolonged periods; serious adverse events; discontinued use; and some, like steroids, are associated with an increased risk of lymphoma\(^12\) and/or death.\(^13\) In population-based studies, approximately 75% of individuals relapse over five years and the most effective agents are only 25–40% effective in maintaining remission over one year.\(^14\)\(^15\) Many of the monoclonal antibody biologics also have short-term side effects (with specific differences associated with
The present investigations are extensions of previous reports on the therapeutic value of Dietzia as a probiotic for an IBD-like disease of ruminants, called Johne disease. This disease, found predominantly in cattle, sheep, and goats has many manifestations in common with Crohn disease including debilitating diarrhea. As is the situation for IBD patients, outside Dietzia therapy, there are no preventive/curative treatments for animals with Johne disease. The etiologic agent of Johne disease is Mycobacterium avium subspecies paratuberculosis (MAP). Infection of cattle with MAP that results in clinical disease, usually at >2 years of age, occurs primarily in utero and in newborns (reviewed in ref. 68). For disease to be manifested in cattle, infection with MAP and immune incited intestinal inflammation are required. Interestingly, MAP is at the center of controversy regarding its role in CD, as well as sarcoidosis, and more recently, in type-1 diabetes mellitus. Blau syndrome, ulcerative colitis and irritable bowel syndrome; the latter two, like CD, are considered to be a result of chronic gastrointestinal inflammation. Although association of MAP with Crohn disease appears to be specific, and all the evidence taken together strongly indicate a causative role, such a role remains to be unequivocally established.

A rarely studied genus of bacteria, Dietzia was found to (a) inhibit growth of MAP (hypothetized to be due to competition for iron) under specific in vitro culture conditions, (b) effectively prevent/delay clinical manifestations of Johne disease in asymptomatic adult cattle and (c) eradicate MAP from calves that are infected in utero and/or as neonates. Based on these findings, plus the many similarities of Johne and Crohn diseases, the study herein was undertaken to assess whether a therapeutic protocol defined for cattle at different stages of infection (see Materials and Methods for definition of the four different stages) might have value for resolution of diarrhea, as well as inflammation, in Crohn patients, irrespective of whether MAP is/is not the etiologic agent. Since bovine biologic agents similar to those used for treating humans are not readily available or characterized, the steroid, dexamethasone was chosen as a means to mimic the human counterpart for controlling/reducing inflammation.

Results

Control of diarrhea in end-stage IV diseased cows. The first experiments were to determine if Dietzia would have any therapeutic value for cows with daily and persistent “pipestream” diarrhea manifestations (at each bowel movement, independent of the number/day); this parameter, being the most easily monitored, defines (and is unique for) end-stage Johne disease (Stage IV), irrespective of in vitro test-parameters (ELISA, AGID, and/or fecal shedding values), weight loss and/or depressed appetite. Six clinically end-stage animals were fed 1x10¹¹ colony forming units (cfu) of freshly prepared, non-frozen, viable Dietzia. For these initial investigations, Dietzia was grown in agar plates, which imposed severe limitations on quantities available. Consequently, many lapses occurred in daily treatment, which resulted in diarrheic relapse. In addition, inactivation of

Figure 1. Photographs of Stage IV, end-stage, diseased cow, Green-4, before and after Dietzia treatment. Photo on the left is prior to treatment and photo on the right is four months post-treatment: note increase in body mass and improved coat appearance.
Dietzia by gamma-radiation also resulted in loss of its clinical benefit. Resuming treatment with viable Dietzia reversed clinical manifestations. In contrast, the 10 Stage IV control animals that were not treated with Dietzia continued with unabated diarrhea and never went into remission. Thus, oral treatment with viable Dietzia effectively controlled diarrhea in Stage IV animals although daily treatment was required to maintain this status. An uninterrupted, daily Dietzia treatment became possible once protocols were developed to grow them in large biofermenters. To balance the abundance of material that would have

### Table 1. Parameters of Dietzia-treated and non-treated Stage II or III paratuberculosis cows

| Cow ID | Breed1 | ELISA I-M-F2 | Fecal I-M-F3 | AGID | Months Dietzia Treatment | Months Survival Post-Initial ELISA |
|--------|--------|--------------|--------------|------|------------------------|----------------------------------|
|        |        |              |              |      |                        |                                  |
| Not treated |        |              |              |      |                        |                                  |
| 33     | J      | 1.5 / 1.8 / 1.8 | 0 / 25 / 25  | --   | 0                      | 6+                               |
| B9     | J      | 1.5 / 2.6 / 2.6 | 0 / 0 / 0    | --   | 0                      | 11                               |
| 29     | J      | 1.6 / 2.2 / 2.2 | 0 / 0 / nd   | --   | 0                      | 15                               |
| 3056   | H      | 1.8 / 3.0 / 7  | 0 / 2 / 2    | --   | 0                      | 20+                              |
| B37    | J      | 2.1 / 3.0 / 3.0 | 1 / 1 / 0    | --   | 0                      | 7                                |
| 266    | J      | 2.3 / 3.4 / 2.3 | 0 / >300 / >300 | +  | 0                      | 14                               |
| 16     | J      | 2.4 / 2.4 / 2.4 | 0 / 0 / 0    | --   | 0                      | 1.5                              |
| H42    | J      | 2.7 / 2.7 / 2.7 | 6 / 6 / 6    | --   | 0                      | 3                                |
| B42    | J      | 2.8 / 4.3 / 4.3 | 8 / >300 / >300 | --  | 0                      | 9                                |
| Mean   |        | 2.1 / 2.8 / 2.7 | Median 0     | 9    |                         |                                  |
| Decreasing ELISA |        |              |              |      |                        |                                  |
| 36     | H      | 1.7 / 1.7 / 1.1 | 0 / 5 / 0    | --   | 11                     | 50+                              |
| 228    | J      | 2.0 / 2.0 / 0.52 | 0 / 0 / 0    | --   | 12                     | 56+                              |
| B70    | H      | 2.0 / 2.3 / 0.53 | 0 / 0 / 0    | --   | 20                     | 34+                              |
| 229    | J      | 2.2 / 2.2 / 0.60 | 1 / 4 / 0    | --   | 12                     | 73+                              |
| 13     | J      | 2.2 / 2.2 / 1.4  | 0 / 0 / 0    | --   | 15                     | 18                               |
| 1734   | H      | 2.2 / 3.1 / 0.52 | 0 / 1 / 0    | --   | 17                     | 50+                              |
| S2     | H      | 3.0 / 3.0 / 1.2  | 0 / 8 / 0    | --   | 26                     | 34+                              |
| Mean   |        | 2.2 / 2.6 / 0.84 | Median 15    | 50+  |                         |                                  |
| Increasing ELISA |        |              |              |      |                        |                                  |
| 212    | J      | 1.5 / 2.0 / 1.2  | 2 / 100 / 100 | +   | 28                     | 28                               |
| R1     | X      | 1.5 / 3.5 / 3.4  | 0 / 39 / 39  | +    | 13                     | 13                               |
| 231    | X      | 1.6 / 2.7 / 1.9  | 0 / 8 / 8    | --   | 14                     | 27+                              |
| 65     | J      | 1.8 / 3.7 / 3.7  | 0 / 30 / 19  | +    | 16                     | 16                               |
| 9030   | H      | 1.9 / 3.1 / 3.1  | 0 / 44 / 44  | --   | 4                      | 4+                               |
| 232a   | H      | 2.0 / 4.9 / 4.9  | 0 / >300 / 5 | +   | 26                     | 26+                              |
| 234    | J      | 2.1 / 4.0 / 4.0  | 0 / 22 / 22  | +    | 12                     | 12                               |
| R38    | H      | 2.3 / 4.0 / 3.8  | 0 / >300 / >300 | +  | 31                     | 31                               |
| 227    | H      | 2.4 / 3.5 / 3.5  | 0 / 0 / 0    | --   | 6                      | 6+                               |
| 21     | H      | 2.4 / 4.3 / 2.7  | 1 / >300 / 78 | +  | 33                     | 33                               |
| Green 8| H      | 2.7 / 4.1 / 3.4  | 213 / >300 / 19 | +  | 7                      | 7                                |
| Green 9| H      | 2.7 / 3.8 / 3.8  | 0 / 225 / 225 | +   | 26                     | 26                               |
| 1826   | H      | 2.9 / 4.3 / 3.8  | 20 / >300 / 80 | +  | 26                     | 26                               |
| R23    | H      | 3.0 / 3.9 / 3.9  | 4 / 42 / 42  | --   | 2.5                    | 2.5                              |
| Green 3| H      | 3.0 / 3.9 / 3.4  | 6 / >300 / >300 | +  | 34                     | 34                               |
| 9238   | H      | 3.0 / 3.6 / 3.6  | 26 / 37 / 0  | +    | 14                     | 14                               |
| Mean   |        | 2.3 / 3.7 / 3.4  | Median 15    | 21   |                         |                                  |

1J, Jersey; H, Holstein; X, Cross; 2ELISA value; I, initial; M, maximum; F, final (within four weeks of demise); 3Fecal, cfu MAP/2 gms; I, initial; M, maximum; F, final (within four weeks of demise); 4Initial ELISA shown in column 3. 5nd, not done; 6Parameters prior to dexamethasone treatment—see Table 2
to be stored frozen (viability decreased one log in six months at
-20°C) versus costs, it was predetermined that the treatment-dose
would be adjusted based on changes in clinical status and not on
changes in any specific sero- or fecal-value. Pictured in Figure 1
(left panel) is an emaciated, Stage IV diseased cow, referred to
as “Green-4,” prior to treatment. The consensus of three local
veterinarians, plus my own assessment based on previous experi-
ence with untreated Stage IV animals, was that the odds of her
surviving two weeks was zero. Figure 1 (right panel) shows her
in remission after four months of daily treatment. Changes in
body mass, milk production, ELISA values and fecal MAP
values are shown at different doses of Dietzia in Figure 2. As shown in
Figure 2A, once started on Dietzia, body mass and milk produc-
tion both increased. Upon attaining remission, the Dietzia dose
was tapered (Fig. 2C), after which she clinically relapsed, lost
weight and produced less milk. She also had a left side displaced
abomasum (LDA)—one of the four stomachs became twisted—
which was corrected non-surgically on 2/10/02. Because of the
relapse and LDA, the dose of Dietzia was increased, and again an
increase in body mass occurred. Being within months of calving,
milking was discontinued (dried off) at this time. After recover-
ing, Dietzia was again lowered; she calved with twins and later
succumbed with end-stage IV clinical disease. At autopsy she was
confirmed to harbor MAP in multiple tissues and the intestinal
pathology was characteristic of paratuberculosis. As shown in
Figure 2B, ELISA values remained essentially unchanged over
the entire treatment period. She became AGID-positive (a sero-
logy test that correlates with a more advanced stage of disease
than ELISA values) at the second test-date and remained so for
the remainder of her life. In contrast, the extent of fecal shedding
was extremely variable, appearing to be associated with the dose
of Dietzia; the higher the dose, the lower the shedding. Changes
in body mass appeared to lag changes in fecal shedding.

Dietzia treatment of animals with Stage II or III disease. The
second goal was to further refine a Dietzia treatment for animals
with Johne disease that could be tested in humans with chronic
diarrheal diseases. Because of variable ELISA values of animals
with low to moderate initial values (ODs ≤ 3), determination of
the longitudinally best-linear fit of values was the most inform-
ative way to assess effectiveness of treatment.83 Such analysis
also reduced the impact of each individual value. Employing this
type analysis, it was previously reported that approximately half
of Dietzia-treated Johne diseased cows had decreasing longitudi-
nal ELISA values, whereas all non-treated cows had increasing
values.83 Figure 3 shows such decreasing ELISA values for seven
cows considered cured by treatment with Dietzia only; however,
as previously reported, not all animals with decreasing values
were cured. For the cured cows, treatment was discontinued
after all paratuberculosis test-parameters became negative. Only
one animal succumbed early (cow 13) due to complications from
milk fever post-calving; all others were terminated at the end of
the study with no evidence of clinical disease [milk fever is due
to inadequate Ca++ absorption from the intestines, compensated
by calcium depletion in muscle that causes an inability to stand
and/or move (potentially causing permanent nerve damage in
limbs and if sufficiently severe, cardiac failure and death)]. The
median treatment time was 15 months and the median survival
from the time they were detected paratuberculosis positive was
>50 months (Table 1), a value not significantly different from
that of paratuberculosis-free animals.83 Survival times with a pos-
tive symbol (+) indicate the animal was terminated for reasons
other than Johne disease; i.e., survival would have been longer
than shown. Because some animals never fecally shed MAP and
others shed only once or twice, shedding values are shown only
in the table. None were shedding when terminated and none
ever became AGID-positive. Moreover, none showed any clinical
symptoms of Johne disease at any time, and at autopsy, cows #13
and #52 were completely devoid of any intestinal inflammation
characteristic of Johne disease and lacked culture/PCR detect-
able MAP in all tissues.

In contrast, the median survival of animals with increasing
longitudinal ELISA values was, (a) 9 months for untreated ani-
mals (all but 33 and 3056 succumbed with end stage disease and
only one survived to became AGID-positive) and (b) 21 months
for those treated [again “months number” with a positive symbol
(+) indicate the animal was terminated for reasons other than
Johne disease]. Median treatment time was again 15 months;
however, unlike the animals considered cured, 12 of 16 event-
tually succumbed with end-stage clinical disease. All 12 were
AGID-positive; three of the four that remained AGID-negative
had very short survival times and the fourth one was terminated
for reproductive reasons with Stage II disease. Thus, even though
animals with increasing ELISA values were not cured, their sur-
vival time was significantly extended.

Figure 2. Longitudinal changes in body weight, milk production, ELISA
values and fecal MAP for Stage IV cow, Green-4. (A) Dashed line is body
weight and solid line is official DHIA weight of milk produced/day.
(B) Solid line is ELISA OD405 nm values and dashed line is fecal MAP.
Symbol (+) signifies a positive AGID. (C) Dose (viable colony forming
units, cfu) of Dietzia.
The initial, maximum, and final ELISA and fecal shedding values are also presented in Table 1. The mean initial ELISA values of the three groups (not treated, treated with a declining ELISA, and treated with an increasing ELISA) were not significantly different (p > 0.35), suggesting that each group, on average, was at a similar stage of disease when treatment was initiated. The final, mean ELISA value of the cured group (declining ELISA) was significantly less than the final means of the other two (p < 0.0001); the latter two were not significantly different from each other (p > 0.05). Also fecal shedding (final) was absent in the cured group, but was detected in most animals in the other groups.

One animal (cow #198) had longitudinal ELISA changes that were biphasic. As shown in Figure 4, during the initial 18 months of treatment, the best-linear-fit curve declined to negative values similar to that found for animals considered cured. However, after discontinuing treatment for nine months, the longitudinal best-linear-fit curve increased similar to that of non-treated paratuberculosis animals and she succumbed seven months after discontinuing treatment for nine months, the longitudinal best-linear-fit curve declined to negative values (<1.4), suggesting the absence of systemic MAP activity independently of that which followed declining MAP levels after treatments with high doses of Dietzia. Results for such dual treatments are shown for individual animals in Table 2 and Figures 5–8.

As shown in Figure 5, April (weighing 800 pounds) relapsed from Stage II (asymptomatic) to Stage IV (severe clinical disease) on 11/25/05 while being treated with Dietzia only. At this stage she was given dexamethasone (2 mg/day IM) for seven days, which reversed the clinical symptoms. She was then maintained on 0.4 mg every other day for two months followed by 0.2 mg every other day for another two months; at which time dexamethasone was discontinued (Dietzia was continued). She remained asymptomatic (Stage II) for an additional seven months at which time she again relapsed (Stage III). Based on previous experience, it was deemed unlikely further treatment would be beneficial and she was terminated. As shown, her ELISA values increased or remained unchanged up until dexamethasone was initiated,

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**Table 2. Dietzia plus dexamethasone treatment of Stage III or IV paratuberculosis cows**

| Cow ID | Breed | ELISA I-M-F | Fecal I-M-F | AGID | Months Dietzia treatment | Dexamethasone post-/pre-Dietzia | Months survival post-initial E* |
|--------|-------|-------------|-------------|------|--------------------------|-----------------------------|-------------------------------|
| 232^1  | H     | 2.0/4.9/3.1 | >300/300    | +    | 34                       | Post                        | 34+                           |
| April^1 | J     | 2.5/3.7/3.3 | 65/300/58   | +    | 20                       | Post                        | 20+                           |
| Tixie^1 | J     | 3.0/3.7/1.4 | 7/300/300   | +    | 17                       | Post                        | 17                            |
| 225^1  | J     | 1.7/1.7/0.93| 33/33/0     | +    | 5                        | Pre                         | 10                            |
| Monica | J     | 3.3/3.3/3.3 | >300^1      | +    | 0.75                     | Pre                         | 1.25                          |

^1J, Jersey; H, Holstein; ^2ELISA values: I, initial; M, maximum; F, final (within 4 weeks of demise); ^3Fecal cfu/2 gm: I, initial; M, maximum; F, final (within four weeks of demise); ^4Initial ELISA values shown in column 3. ^5Terminated with Stage III disease; ^6Died from accident; ^7Actual value was 9,200 cfu after correction for a 1:100 dilution.
at which time, antibodies detected by ELISA declined. AGID antibodies detected at the first five test points became undetectable by the last two, further supporting a curtailment of humoral immune activity to MAP. Fecal shedding increased to a stable level and only declined after dexamethasone was discontinued and Dietzia was increased to >9 x 10^11 cfu.

Figure 4 shows the effects of Dietzia only at Stage II, and later (at Stage III) in combination with dexamethasone for cow #232, a Holstein weighing 1200 pounds. ELISA values increased steadily during early Dietzia treatment; reached a plateau; and declined after dexamethasone treatment was initiated. AGID became positive and eventually returned to negative after extended treatment with dexamethasone. The induction dose of dexamethasone (5 mg/day) that resulted in remission was higher than that found for April, most likely due to body weight differences. Fecal shedding was low during Dietzia treatment, but dramatically higher while on dexamethasone. She was also terminated when clinical Stage III disease, presumed irresolvable, reoccurred.

Figure 5 shows the effect of treating Trixie, a Jersey weighing 1000 pounds with lifetime (Stage III) intermittent diarrhea, with Dietzia daily, and dexamethasone only during relapses. As shown, fecal shedding was not controlled by the dual treatment. She was AGID-positive at the first test-point and was negative at all subsequent times. Her ELISA values steadily declined, and two weeks prior to her untimely accidental death five months post-treatment. Monica, with Stage IV clinical disease, was given 5 mg dexamethasone daily for two weeks, which alleviated pipetream diarrhea and depressed appetite, and then discontinued. Thereafter, a daily dose of 10^13 cfu Dietzia was initiated. She succumbed two weeks later with normal manure consistency and appetite, suggesting that intestinal damage was so advanced that treatment was ineffective. Unfortunately, an autopsy was not an option because she died on a summer Saturday when the university diagnostic laboratory was closed.

Safety of Dietzia. Both paratuberculosis-free and -positive adult cattle reported herein and previously81-83 showed no adverse effects, some for over 3½ years, that could be attributed to long-term Dietzia treatment; moreover, five paratuberculosis-free cows fed Dietzia for up to a year never became sero-positive for Dietzia.81 In addition, none of 46 newborn calves (36 from paratuberculosis-negative and 10 from -positive dams) fed daily with Dietzia for 60 days became positive for any parameter (including antibody) associated with Johne disease or showed any adverse long-term side-effects (Click, unpublished). All calves gained weight and matured indistinguishably from untreated calves. During their lives of up to 70 months, there were no signs of disease or sickness related to treatment. Further evidence of safety was demonstrated by IP injection of viable Dietzia into normal C.B20 and immunoincompetent C.B17 scid/scid mice. All of the parameters monitored (weight, diarrhea, reproduction) in the treated mice were indistinguishable from those of untreated mice.

Any impact of oral consumption of Dietzia related to food chain safety was also examined. The amount of viable Dietzia in LTLT pasteurized milk was <1 cfu/L, compared to the unpasteurized, spiked original, which still possessed 1x10^8 cfu/ml. Further, Dietzia was undetectable in milk from three Dietzia-treated cows.

Discussion

Based on similarities of Crohn and Johne diseases, the investigations presented herein with adult cattle were undertaken to evaluate whether a protocol employing Dietzia, a bacterium with MAP growth inhibitory activity in culture, and one found therapeutic when used as a probiotic, short- and long-term, for cattle, could be adapted for use in IBD (especially CD) patients. The ultimate goal was two-fold: (1) ameliorate diarrhea in the short-term, and (2) define a protocol with potential long-term therapeutic (curative) value. Findings with end-stage (Stage IV) Johne diseased animals indicated that diarrhea was ameliorated by a daily treatment of viable Dietzia, but not by gamma-radiated Dietzia or by intermittent treatment; suggesting viable Dietzia functioned as a conventional probiotic. Clinical remission of Stage IV diseased animals (example, cow Green-4) was achieved with uninterrupted high doses of Dietzia, whereas at lower doses or intermittent dosing, relapses occurred. This oscillation was most likely due to both a probiotic effect and a reduction (killing?) of the
etiological agent, MAP per se. The findings suggest that a consistently high dose of Dietzia (>10^12 cfu) over the entire duration of treatment may have resulted in even better therapeutic outcomes; however, at the time it was deemed more important to investigate effectiveness of specific doses at different stages of disease.

To be an effective, long-term treatment for Johne disease, two processes must be curtailed: (a) inflammation of the intestine, presumed to be due to an immune response to MAP, and (b) eradication of MAP, the etiological agent stimulating immune reactivity. Previous and present results indicate that the clinical efficacy of Dietzia treatment was directly associated with longitudinal changes of ELISA values; maximum survival and cures were associated with decreasing ELISA values. Treated animals with increasing values were not cured, but median survival was extended relative to those not treated.

The results obtained with cow #198, in which the ELISA best-fit analysis showed a biphasic curve, raise a question regarding animals defined as cured. Initial treatment of 198 resulted in declining best-fit ELISA values similar to those of animals considered cured. The ELISA-negative values were maintained for nine months post-treatment. At this time, the ELISA values began to increase at a rate similar to that of non-treated animals and she eventually succumbed with end stage clinical disease, which was confirmed at autopsy. Thus, was disappearance of disease-associated test-parameters actually a consequence of MAP elimination (cured), or was the undetected level of fecal MAP simply insufficient to induce antibody synthesis? Or would have positive parameters reappeared in the eight animals considered cured had they survived longer? Irrespective of the answer, a number of cows (#s 228, 229, 36, 1734) survived more than 50 months after initiation of treatment favoring the absence of MAP. The probability of recurrence cannot be ruled out for those with shorter survival times, although cows #13 and #52 were devoid of any evidence suggestive of paratuberculosis at autopsy. To distinguish between the alternatives is likely untestable because of limited lifespans; indeed, is it even relevant for dairy cattle because of their short productive lifetimes (average of 4.5 years)? More importantly, cures obtained with Dietzia were unlike the palliative benefits described for paratuberculosis goats and cattle that were treated with anti-mycobacterials.

Other unanswered questions are: Would continued treatment of cow #198, after becoming negative for paratuberculosis parameters, prevent recurrence? Why was a daily dose required to achieve beneficial results and why were all cows treated with Dietzia not cured. Possible explanations include: (a) the bovine intestine is not an optimal environment for Dietzia since it grows best in vitro at 29°C (bovine body temperature is 39°C), (b) Dietzia requires fructose as its carbon source and it has a rather slow generation time of 3–4 h, (c) different bovine resistance/susceptibility genetics, (d) genetics of different subtypes of MAP present on the farms the cows came from, (e) use of insufficient amounts of Dietzia, and/or (f) differences in intestinal integrity present at the start of treatment. As a means to (a) enhance cures/survival times, (b) uncouple Dietzia’s reduction of MAP fecal excretion from subsequent antibody decline, and (c) more closely mimic protocols presently employed to treat IBD patients, a pilot investigation was undertaken with dexamethasone, a glucocorticoid shown to be immunosuppressive in cattle, in combination with Dietzia. As expected, dexamethasone effectively reduced both serum ELISA and/or AGID detectable antibodies specific for MAP in all animals that were dually treated. However, such improvement was accompanied by an exacerbation of the reduced and/or maintained low levels of fecal shedding associated with high doses of Dietzia; a reduction that normally occurred prior to any curtailment of specific antibodies. In addition, the one animal treated extensively with dexamethasone developed an ulcer, similar to fistulas present in Crohn patients, which led to her death. Even though the mechanism by which Dietzia curtailed MAP remains unknown, because it, unlike indigenous probiotics, prevented growth of the etiological agent (MAP) in culture, hypotheses being considered are: (a) close intracellular (macrophage?) encounters of MAP and Dietzia could easily be enhanced by cross-reactive antibodies specific for epitopes they share (Richards, personal communication and Click, unpublished), thus allowing enhanced opsonization of Dietzia/MAP by phagocytes possessing MAP/Dietzia, (b) Dietzia, when in close proximity to MAP, either inhibits its growth via competition for a nutrient(s), or directly kills it, (c) steroid disruption of normal phagocytic cellular activity/integrity could impair potential MAP killing, and (d) in the absence of co-infection or macrophage impairment, MAP levels would not be curtailed. The most intriguing aspect of these postulates is that for Dietzia to effectively reduce and eliminate MAP, immune reactivity (with an emphasis on humoral) to MAP is essential, even though such activity in the absence of Dietzia is not beneficial/curative. Thus, the perplexing problem is: How can inflammation at the mucosal barrier be curtailed sufficiently without impacting the immunological reactivity necessary to eliminate MAP? In many cases, curtailment could take a very
Mapotic therapy can be discontinued once in vitro test-parameters of infection; might be an alternative. Nosuppressive treatment prior to treating with Dietzia (#255), was safe, with no apparent lifetime side effects. Three years and injection into normal or immunodeficient mice on daily therapy for their lifetime; clinical disease manifested by severe diarrhea, must be continued for up to a long time, during which damage to the intestines may become so extensive that recovery is improbable, as appears to be the case for the Jersey cow, Monica (she was shedding an astonishingly actual count of 9,200 cfu/2 gms fecal material). Such a scenario may be extremely relevant for immunosuppressive biologic regimens presently used for IBD patients; i.e., can cures ever be attained using only immune modulating protocols? A short-term immunosuppressive treatment prior to treating with Dietzia (#255), might be an alternative.

In summary, the results presented herein define:

a) a successful treatment and eradication of asymptomatic MAP infection with Dietzia, when used as a probiotic;

b) a successful Dietzia treatment for symptomatic and otherwise terminal MAP infection;

c) that the Dietzia treatment for cows with symptomatic disease is enhanced by short treatment-intervals with dexamethasone;

d) in cows with asymptomatic MAP infection, daily probiotic therapy can be discontinued once in vitro test-parameters of MAP infection become negative, while animals with end-stage clinical disease manifested by severe diarrhea, must be continued on daily therapy for their lifetime;

e) that daily administration of viable Dietzia to cows for up to three years and injection into normal or immunodeficient mice was safe, with no apparent lifetime side effects.

Similarity of Human and Bovine Gastrointestinal Diseases

Crohn disease, ulcerative colitis, and IBS are generally accepted to be a result of chronic gastrointestinal inflammation; the present discussion will, however, focus primarily on Crohn disease. Environmental factors play a role in the development, and prevalence varies with time, geography, socioeconomic conditions, and occupation. The incidence is more common in urban than in rural areas and in highly developed industrialized countries than in less developed tropical countries. CD is a chronic debilitating disease characterized by an unpredictable disease course, potential complications such as fistulas, and the frequent need for surgery. Ultimately, alterations in the composition of the intestinal flora may promote bacterial invasion of the mucosa and predispose patients to chronic inflammation. Inflammation is characterized by an inductive or initiation phase followed by a sustained response that ends when there is resolution of the process(es). This abnormal response may have two origins: an increased proinflammatory response to a bacterial component or a decreased regulatory response, which may lead to an excessive effector immune response.” As a consequence, an unregulated immune response develops to, at least in part, microbial antigens. Although the genetic make-up of an individual confers risk to develop CD, specific susceptibility genes (some in common with those present in cattle) are neither required nor sufficient.

Factors that influence the choice of therapy in Crohn disease are multifactorial. Conventional treatments are directed primarily at achieving symptomatic relief, preventing relapses, and steroid sparing; i.e., treatments are directed at controlling inflammation directly. They are not directed at the elusive undefined etiologic agent(s). Immune modulation is most efficacious if implemented during early disease and tends to be less effective for late stages. At present there are no conventional treatments that result in cures or permanent remissions. It is postulated that microbial species in non-inflamed tissue, prior to detection of inflammation, hold clues to microbial pathogenesis, primarily because initiation of disease process(es) precedes clinical manifestations. Microorganisms implicated in CD (some for UC), besides MAP, include Candida, enteroadherent-invasive E. coli strains, Bacteroides and Fusobacterium species and even normal commensal non-pathogenic species. Perhaps the most important question to ask regarding a role for any of these organisms is: Are they etiologic agents? Are they merely perpetuators of disease once events leading to disease are initiated? Or are they merely opportunistic bystanders? Presently, association of these different organisms with disease best fit the latter two alternatives, and not etiologically initiators, primarily because the time of initial insult remains unknown. If the initiation event, relative to detectable parameters associated with disease, is similar to one (in utero) ascribed for Johne disease of cattle, it very well may occur years prior to onset of detectable disease, clearly way before identification of intestinal microbes present at the time of diagnosis of clinical disease.

It has been proposed that a therapy directed against both a bacterial etiology agent and against inflammation may be a more fruitful approach to controlling Crohn disease than conventional mono-therapies. Therapeutic strategies aimed at restoring the host microbe balance at the intestinal mucosa by fecal bacteriotherapy or with probiotics may prove superior to, with far less side-effects, treatments that broadly suppress inflammation and/or innate immunity or may prove to be an exceedingly
beneficial adjunct therapy. An excellent example of this is that combination therapy (probiotics as one agent) improved cure rates for Helicobacter pylori.120

Proposed Adaption of Dietzia Treatment to Crohn Patients

Treatments for Crohn and Johne diseases are most effective when started at early stages of disease. Based on findings that human gastrointestinal,46-48 hepatic63 and other diseases121 were successfully treated with organisms functioning as probiotics, it is surprising that probiotics are not effective for CD.58,122,123 In fact, Lactobacilli were completely ineffective.124,125 However, because this group of IBD patients has the highest incidence of MAP infection, MAP could be a useful target for mitigating disease, irrespective of whether it is/ is not the causative/perpetutive etiology agent. This fact, in conjunction with the presumed dual activity—probiotic function (ameliorate diarrhea) plus direct inhibition of MAP growth (in culture)—of the non-indigenous probiotic, Dietzia, is sufficiently compelling to warrant undertaking a clinical Dietzia trial with Crohn patients, especially those documented to harbor MAP. Because viable Dietzia had no adverse side effects and was nonpathogenic when administered orally to adult cows or newborn calves (Click, unpublished), as well as when injected IP into mice, it is anticipated that it will also be found safe for oral consumption by humans. Following is a proposed adaption of the cattle protocol for testing in a clinical trial.

1. A standard, constant (not variable as used herein for cattle) Dietzia dose of $10^{11}$ viable cfu per 100 lbs. body weight would be orally administered daily. Pass-through would be monitored.

2. Two groups of patients would be enrolled—those documented to be infected with MAP and those that are MAP-test-negative (even though they may also be infected). Appropriate randomization of non-treated and treated patients would be done to meet statistical criteria.

3. Parameters to be monitored126,127 include serum antibody, quantitative determination of MAP in blood, and standard clinical assessment. Side effects would also be monitored.

4. Daily treatment would continue until all in vitro test parameters become negative and then each group would be divided into treatment terminated, or treatment continued (undefined time).

Materials and Methods

Experimental design. The primary goal was to refine a previously published therapeutic protocol used to treat adult paratuberculosis cows for testing in humans. To this end, a roughly 4:1 ratio of paratuberculosis-free and -positive animals (see below for definition of each), under St. Croix Valley Farm (SCVF) ownership and management, were housed together in a tie-stall facility (each animal tied in their own space) as a single dairy herd. All aspects of the research were conducted using standard operating conditions of a normal dairy farm. At any given time, the herd was comprised of 50–60 females. Once a paratuberculosis parameter was detected positive, treatment was or was not initiated and then various paratuberculosis parameters were monitored over the animal’s remaining lifetime. It was predetermined for this study, based on cost considerations and as a means to define effective doses for different stages of disease, that the dose of Dietzia would be adjusted for each animal based only on changes in clinical status and not on changes in any paratuberculosis-specific test parameter. Since many animals showed clinical relapse, the dose was empirically varied based on body mass and severity of clinical disease throughout the study. Emaciated animals were defined as having end-stage clinical Johne disease based on the presence of both “pipestream” diarrhea and depressed appetite. Local veterinarians humanely euthanized recumbent, emaciated and/or cachetic end-stage animals by intravenous injection of a sodium pentobarbital solution (Fatal Plus, 6 gm/ml, Vortech Pharmaceuticals, Dearborn, MI) at 1 ml/4.5 kg body weight when they no longer could get up and stand on their own. Animals that showed potential life-threatening non-Johne disease ramifications were sent to slaughter. All other aspects of the project were handled by normal dairy procedures.

Animals. Most animals in the study were adult dairy cows in their second through fourth lactation. Severity of disease was defined by the following classification:128 animals that were asymptomatic, and test-parameter negative were classified at Stage I, those asymptomatic, and either ELISA and/or fecal positive were classified as being at Stage II. Stage III animals showed signs of early clinical disease and were either ELISA and/or fecal positive, whereas, Stage IV animals were severe, end-stage clinically, irrespective of whether they were ELISA and/or fecal negative/positive (almost all were fecal shedding). Of the original Stage II and III diseased cows,83 detected positive at dry-off (two months prior to their predicted calving date, milking was discontinued) by paratuberculosis-specific ELISAs (determined by the owners
and their veterinarians), purchased from seven local, well-managed, moderately high-prevalence herds over a five-year period, 32, plus an additional 18 Stage IV and 3 Stage III cows not in the original group, are included in the present study. The final classification of an ELISA-positive animal as having Johne disease was based on whether it eventually (a) tested positive for a different parameter (fecal shedding or serum AGID), (b) developed end-stage (Stage IV) clinical disease, (c) was determined to have paratuberculosis via complete autopsy, and/or (d) only as a last resort, tested ELISA-positive multiple times. As reported previously, when an ELISA was positive (OD > 1.4), it was considered an accurate indicator of paratuberculosis. For the present experiments, animals were divided into two distinct groups; those with Stage II or III disease with ELISA values equal to or less than 3.0, and those with Stage IV disease (end-stage clinical disease). As the purpose was to assess the effect of intervention rather than confirm the findings of others that non-treated paratuberculosis animals eventually succumb with clinical disease, more Stage II and III animals were in the treated group (n = 26) than in the non-treated group (n = 9). Stage IV animals were either treated (n = 8) or not treated (n = 10).

**Dietzia.** Dietzia (partially characterized and originally misclassified as *Mycobacterium gardonae*) was isolated from fecal material of a paratuberculosis soro- and fecal-positive cow. It was reclassified as Dietzia based on its 16S rRNA sequence (performed by MIDI Labs, Inc., Newark, DE), which is considered the gold standard for bacterial identification. Growth requirements were defined in agar plates. Ultimately, Dietzia was grown for 3–4 days at 29°C under contract in a 75-liter fermenter at the University of Minnesota Biotechnology Institute (St. Paul, MN) in fructose-supplemented tryptic soy broth. Batches were centrifuged, washed, and concentrated 20-fold prior to storage in 45 ml aliquots at -80°C (long term) or -20°C (short term). New lots were prepared as needed, approximately every 2–3 months. The number of colony forming units/ml was determined prior to use. Once thawed, aliquots were maintained at 4°C for up to 10 days only. Dietzia treatment was always initiated after an animal was detected ELISA-positive. Based on preliminary dosage experiments, small cows (Jerseys and Jersey x Holstein crosses), and large cows (Holsteins and Guernseys) were initially treated by supplementing their morning feed with Dietzia at a minimally effective daily dose of 2–3x10^11 and 4–5x10^11 cfu, respectively. The dose was increased if an animal showed clinical signs of disease and then lowered if remission was achieved (see above reason). For a few specific treatments, Dietzia was inactivated by 10 Gy gamma-radiation from a cesium source. Based on the ineffectiveness of gamma-radiated Dietzia, non-treated animals were not given any placebo-type growth medium or other inert material.

The sensitivity of Dietzia to LTLT (Low Temperature, Long Time, 145°C for 30 min) pasteurization was determined to assess the possibility of viable Dietzia getting into the food chain through the milk supply. Milk from non-treated paratuberculosis-free animals was spiked with viable Dietzia to achieve 1x10^8 cfu/ml. One half of this milk was pasteurized and the other half served as the control. The amount of viable Dietzia in each was then determined. In addition, milk from three Dietzia-treated cows was cultured for Dietzia. The Dietzia culture assays were performed by Encore, LLC (Minneapolis, MN).

**Body and milk weights, serum and fecal protocols.** Monthly changes in body weights and daily production of milk (official monthly DHIA milk weights) were initially used to monitor the progression of disease; but were discontinued because changes in each paralleled one another and because fecal composition and reduced feed intake were found to better and earlier define onset of impending clinical disease. Fecal material collected directly from the rectum using individual disposable gloves and blood obtained aseptically from the tail vein were transferred to sterile containers, coded, and sent chilled on the day of collection to Allied Monitor, Inc. (Fayette, MO). Allied Monitor is a USDA- and NVSL-approved laboratory that specializes in assays for Johne disease. The majority, but not all, fecal and serum samples were obtained concurrently. All serum ELISA and AGID assays and fecal MAP cultures were performed upon receipt. Assessment of the validity, sensitivity and specificity of assays (ELISA, AGID, fecal culture) was reported previously. The ELISA for antibodies specific for MAP was performed using a crude, soluble, MAP protoplasmic antigen prepared by Allied. Test sera were preabsorbed with *Mycobacterium phlei*. Split-sample repeatability, as well as duplicate samples, varied less than 5 percent of the mean. The content of each well was read at a single wavelength (405 nm). ELISA values were calculated by dividing the test-sample OD by a value equivalent to ¼ the OD of a standard reference positive serum (range 0.13–0.14), and interpreted as follows: Negative ≤1.4 OD and Positive >1.4. Allied’s classification of ELISAs as Negative (≤1.4 OD), Suspect (1.5 to 2.0 OD) and Positive (>2.0 OD) was reinterpreted based on the fact that 11 of 14 animals with initial “suspect” serum ELISA values eventually became either fecal shedders and/or succumbed with end-stage clinical disease. Of the remaining three, one had multiple ELISAs >2.0, one was Dietzia-treated and became negative for all parameters, and one, not treated, was terminated for unresponsive mastitis three months after initiation of treatment. Therefore, the “suspect” category was not used and all animals with serum ELISAs >1.4 were classified paratuberculosis-positive.
Postmortem analysis. As an additional means to document Johne disease status, pathological postmortem analysis and culture-determination of MAP in tissues was done on randomly chosen cows at the University of Minnesota Veterinary School Diagnostic Laboratory (St. Paul, MN). Summation of the University’s standard basic necropsy, tissue histopathology of multiple organs, culture/PCR, bacteriology, parasitology, serology and molecular diagnostics was used to confirm positive/negative status only; it was not intended to define specific aspects, category of disease, or be compared to ante-mortem parameters.

Safety studies. In addition to adult cattle that were treated daily with Dietzia, some for over 3.5 years, the safety of Dietzia as a probiotic was tested on 46 newborn calves—10 from paratuberculosis-positive dams and 36 from -negative dams. A single oral dose of 1x10¹¹ cfu of viable Dietzia was administered, raising as replacements for the milking herd, were monitored over their lifetime, some over 70 months, for any adverse effects related to the Dietzia treatment.

The safety of Dietzia was also assessed using inbred, conventional CB.20 mice and immune-incompetent CB.17- scid/scid mice. They were housed at the UWRF accredited facilities and were under the supervision of the Local University Staff Veterinarian. Four male and four females (one of each sex per cage) were injected IP with 1x10⁶ cfu viable Dietzia. Four of each sex were not injected and served as controls. The mice were monitored for 6–12 months for any signs of disease (weight loss, diarrhea and reproductive problems). No discomfort, distress, pain or injury was observed outside the initial IP injection of Dietzia.

Statistical methods. Survival time was defined as the number of months from the first detected positive ELISA value (or initial ELISA test for negative animals) until their demise. Linear longitudinal best-fit analysis was used to estimate trends in ELISA values for each animal. The student’s t-test was used to assess differences in mean ELISA values. For all comparisons, p-values less than 0.05 were considered statistically significant.

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Conflict of Interest

The author is a partner in Altick Associates, in SCVF, as well as a member of Paralab, LLC, which is the assignee of a patent application covering the Dietzia technology presented in this paper.

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