Research Notes

Evaluation of the bacterial diversity in cecal contents of laying hens fed various molting diets by using bacterial tag-encoded FLX amplicon pyrosequencing[1]

T. R. Callaway,*[2] S. E. Dowd,† R. D. Wolcott,‡ Y. Sun,‡ J. L. McReynolds,* T. S. Edrington,* J. A. Byrd,* R. C. Anderson,* N. Krueger,* and D. J. Nisbet*

*Food and Feed Safety Research Unit, Southern Plains Agricultural Research Center, Agricultural Research Service, USDA, College Station, TX 77845; †Livestock Issues Research Unit, Lubbock, TX 79403; and ‡Medical Biofilm Research Institute, Lubbock, TX 79410

ABSTRACT Laying hens are typically induced to molt to begin a new egg-laying cycle by withdrawing feed for up to 12 to 14 d. Fasted hens are more susceptible to colonization and tissue invasion by *Salmonella enterica* serovar Enteritidis. Much of this increased incidence in fasted hens is thought to be due to changes in the native intestinal microflora. An alternative to feed withdrawal involves feeding alfalfa meal crumble to hens, which is indigestible by poultry but provides fermentable substrate to the intestinal microbial population and reduces *Salmonella* colonization of hens compared with feed withdrawal. The present study was designed to quantify differences in the cecal microbial population of hens (n = 12) fed a typical layer ration, undergoing feed withdrawal, or being fed alfalfa crumble by using a novel tag bacterial diversity amplification method. *Bacteroides*, *Prevotella*, and *Clostridium* were the most common genera isolated from all treatment groups. Only the ceca of hens undergoing feed withdrawal (n = 4) contained *Salmonella*. The number of genera present was greatest in the alfalfa crumble-fed group and least in the feed withdrawal group (78 vs. 54 genera, respectively). Overall, the microbial diversity was least and *Lactobacillius* populations were not found in the hens undergoing feed withdrawal, which could explain much of these hens' sensitivity to colonization by *Salmonella*.

Key words: bacterial diversity, molting, laying hen

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INTRODUCTION

To initiate a new egg-laying cycle, aging hens in the United States are induced to molt, which increases both egg production and size, and has been induced traditionally through feed withdrawal (**FW**; Bell, 2003). The immune system of birds is inhibited by the stress of starvation, making them more susceptible to colonization by food-borne pathogens, such as *Salmonella enterica* serovar Enteritidis (Holt, 1993; Klasing, 2007). In recent years, alternative molting strategies to FW, such as feeding alfalfa meal, have been shown to have positive effects on health and egg safety by reducing *Salmonella* Enteritidis colonization (Corrier et al., 1997; Landers et al., 2005). This reduction in *Salmonella* is thought to be due, at least in part, to maintaining more normal intestinal microbiota.

Despite more than 50 yr of research into the microbial ecology of the gastrointestinal tract, the gut remains largely a "black box." Recent developments in molecular methodologies have utilized rapid sequencing technologies such as pyrosequencing to evaluate the microbial diversity of the gut quantitatively (Roesch et al., 2007). The present study was designed to quantify differences in the cecal microbial population of chickens fed a typical layer ration, undergoing FW, or being fed alfalfa crumble, using a novel tag bacterial diversity amplification method (**bTEFAP**). We hypothesized that the cecal microbial diversity of hens molted by feeding alfalfa meal would be greater than that of hens molted by FW.

MATERIALS AND METHODS

Single Comb White Leghorn hens, 75 to 80 wk of age, were obtained from a local commercial laying flock and

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[2]Corresponding author: Todd.Callaway@ars.usda.gov

were group housed before the initiation of this study for an acclimation period (described below). All hens were tested for *Salmonella* by cloacal swab and were found to be negative by using the enrichment and plating methods typically used in our laboratory. Briefly, samples were incubated in tetrathionate broth at 39°C for 24 h, followed by a secondary enrichment incubation in Rappaport-Vassiliadis broth at 42°C for 24 h, which was followed by streak-plating on brilliant green agar (Difco Inc., Sparks, MD) to determine the presence of *Salmonella* spp.

Hens were placed in individual wire layer cages and given free access to water and an unmedicated corn- and soybean meal-based layer ration that met NRC recommendations (NRC, 1994). Hens were allowed to acclimate to the wire layer cages for 2 wk and were exposed to a 8L:16D photoperiod for 1 wk before dietary change or feed removal. This light schedule was continued for all 12 d of the study. Four hens were randomly assigned to each of 3 treatment groups: 1) FW, 2) fully fed, and 3) 100% alfalfa crumble. The full-feed ration was a commercial layer ration previously used in our studies (Dunkley et al., 2007) and was the same ration used in the acclimation period. The alfalfa crumble diet used in this study contained >24% crude fiber and 17% CP, and had a low ME (1,200 kcal/kg). Treatment diets were applied to each treatment on d 1 of the molt at the same time feed was removed from hens in the FW group. Treatments were administered for 12 d to coincide with the time period that hens in the FW group were deprived of feed. Hens in all treatment groups were provided access to water ad libitum. On d 12, all hens were humanely killed. The ceca were excised aseptically on necropsy and squeezed into sterile tubes, pooled, and gassed with O_2-free CO_2 and then sealed, stored on wet ice, and shipped overnight for analysis. Fresh cecal contents contained approximately 10^{10} to 10^{11} cells/mL of total culturable anaerobes, based on serial dilution in anoxic-reinforced clostridial broth (Callaway et al., 2002). None of the fresh cecal contents in this study tested positive for *Salmonella* on enrichment.

bTEFAP Analysis

Bacterial tag-encoded FLX amplicon pyrosequencing, which is based on a partial ribosomal amplification followed by pyrosequencing (Dowd et al., 2008) was originally described by Dowd et al. (2008). Total genomic DNA was extracted from fecal samples by using a QIAamp stool DNA mini kit and the methods recommended by the manufacturers (Qiagen, Valencia, CA).

RESULTS

Bacteria were detected in all birds and across treatments. In total, 107 genera were represented, but only 8 genera were found in the ceca of all 12 hens (Figure 1). The different diets did create some apparent shifts in the microbial population of the ceca. Hens that were fed a typical full ration contained 64 genera of bacteria, birds fed an alfalfa crumble ration contained 78 genera, and birds that underwent FW contained only 59 genera (Figure 1).

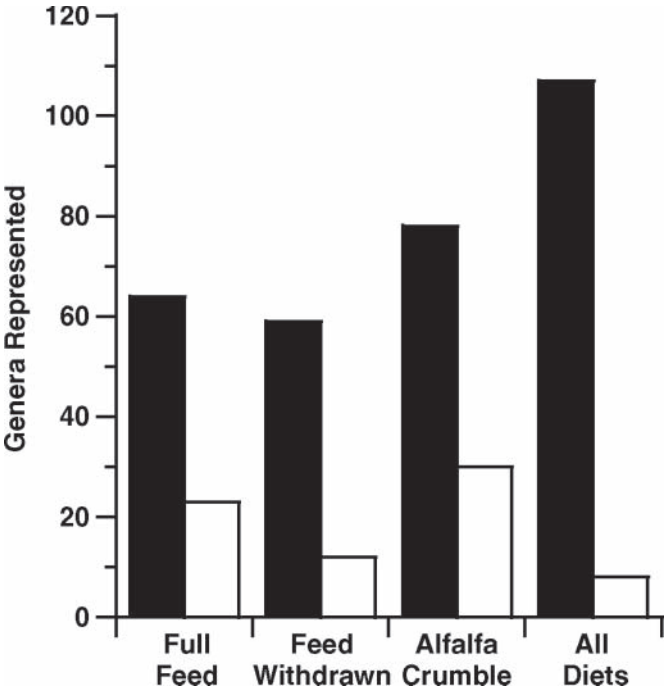

Figure 1. The number of bacterial genera detected in the ceca of laying hens (n = 12) being fed a full-feed ration, undergoing feed withdrawal, or being fed an alternative molting ration of alfalfa meal crumble. Closed bars indicate the number of genera isolated from each treatment; open bars indicate the number of genera that were isolated from all (n = 4) hens in each treatment group. The "all diets" column represents the number of genera present and the number of genera isolated from all hens (n = 12) across diets.

The most common genus detected in the ceca across all birds was *Bacteroides*, which accounted for >37% of the total reads; other genera found in the ceca were *Prevotella* (formerly classified as a *Bacteroides*), *Clostridium*, *Phascolarctobacterium*, and *Ruminococcus*. *Salmonella* spp. were not isolated on plates from any hens, but were detected by bTEFAP from all 4 hens in the FW group and from no birds in any other group (Table 1). However, *Campylobacter* was detected in 2 of the fully fed birds and in 1 of the alfalfa crumble-fed birds (data not shown). Across all 3 diets, 19 genera were found in the cecal populations of all 3 treatments (Table 1). The populations of *Bacteroidales*, *Eubacterium*, *Enterococcus*, *Phascolarctobacterium*, *Firmicutes*, and *Veillonella* were decreased by at least 50% in FW hens compared with fully fed and alfalfa crumble-fed hens. In the FW group, the proportion of cecal populations of *Anaerobiospirillum*, *Ruminococcus*, and *Desulfovibrio* increased 2-fold or greater compared with the fully fed and alfalfa crumble-fed groups. Genera that were uniquely present in hens undergoing FW were *Salmonella* and *Afipia*; the genera *Lactobacillus*, *Thauera*,

Table 1. The 30 most prevalent genera isolated from ceca of molting laying hens in each of the 3 dietary treatments (n = 4 hens/treatment)[1]

Species	Occurrence (n)	Hens containing (n)	Proportion of microbial population (%)	SD
		Full feed		
Bacteroides	3,627	4	39.78	19.71
Prevotella	1,337	4	15.18	7.52
Bacteroidales	685	4	7.65	3.98
Enterococcus	239	4	2.64	1.45
Clostridium	251	4	2.57	1.50
Eubacterium	192	4	2.06	0.73
Lactobacillus	182	4	1.86	1.93
Phascolarctobacterium	165	4	1.80	1.05
Porphyromonas	133	4	1.49	0.58
Dysgonomonas	129	4	1.38	1.25
Fusobacterium	128	4	1.36	1.62
Porphyromonas-like	117	4	1.30	0.99
Helicobacter	113	4	1.20	0.78
Ruminococcus	111	4	1.16	0.86
Saprospira	78	4	0.90	0.28
Limnobacter	81	4	0.87	0.46
Oscillospira	71	4	0.79	0.14
Faecalibacterium	70	4	0.74	0.36
Ochrobactrum	65	4	0.72	0.17
Environmental	67	4	0.71	0.34
Alistipes	64	4	0.67	0.29
Desulfovibrio	42	4	0.46	0.24
Thauera	26	4	0.30	0.25
Candidatus	212	3	2.80	1.93
Anaerobiospirillum	124	3	1.65	0.79
Firmicutes	91	3	1.50	0.57
Veillonella	90	3	1.30	0.48
Pectinatus	64	3	0.91	1.08
Rhodocyclus	59	3	0.90	0.15
Megamonas	29	3	0.43	0.04
		Feed withdrawal		
Bacteroides	6,451	4	44.55	26.49
Salmonella	1,207	4	5.20	4.61
Prevotella	664	4	4.07	3.32
Anaerobiospirillum	657	4	3.71	1.60
Clostridium	467	4	3.09	1.90
Ruminococcus	322	4	2.55	2.43
Desulfovibrio	373	4	2.51	0.64
Limnobacter	203	4	1.87	1.12
Phascolarctobacterium	109	4	0.92	0.90
Rhodocyclus	107	4	0.91	0.71
Oscillospira	75	4	0.61	1.03
Aminomonas	55	4	0.48	0.27
Cytophaga	1,805	3	14.15	11.24
Bacteroidales	222	3	3.46	3.32
Dysgonomonas	241	3	3.02	2.39
Desulfomonas	188	3	2.08	0.71
Eubacterium	164	3	1.45	1.22
Mucispirillum	204	3	1.36	0.93
Enterococcus	132	3	1.13	0.79
Alistipes	42	3	1.10	1.32
Firmicutes	33	3	0.59	0.07
Faecalibacterium	49	3	0.51	1.29
Veillonella	42	3	0.42	1.32
Sporobacter	32	3	0.31	1.36
Pectinatus	21	3	0.30	1.36
Anaerofilum	14	3	0.25	0.19
Sutterella	26	3	0.19	0.23
Roseospira	398	2	4.22	3.54
Afipia	169	2	1.39	1.25
Fusobacterium	81	2	0.71	1.36
		Alfalfa crumble		
Bacteroides	2,913	4	27.39	13.54
Bacteroidales	1,280	4	12.54	5.98
Prevotella	685	4	6.90	6.75
Porphyromonas	519	4	4.94	2.62
Clostridium	465	4	4.60	2.58

Continued

Table 1 (Continued). The 30 most prevalent genera isolated from ceca of molting laying hens in each of the 3 dietary treatments (n = 4 hens/treatment)[1]

	Alfalfa crumble			
Species	Occurrence (n)	Hens containing (n)	Proportion of microbial population (%)	SD
Spirochaeta	482	4	4.53	1.79
Eubacterium	464	4	4.50	1.88
Enterococcus	289	4	2.67	0.97
Unknown	249	4	2.36	0.70
Phascolarctobacterium	237	4	2.04	1.49
Veillonella	169	4	1.54	0.46
Ochrobactrum	176	4	1.50	0.81
Firmicutes	151	4	1.46	0.47
Helicobacter	128	4	1.35	0.59
Lachnospira	141	4	1.25	1.00
Treponema	125	4	1.24	0.31
Anaerobiospirillum	116	4	1.18	0.48
Porphyromonas-like	119	4	1.16	0.92
Limnobacter	114	4	1.03	0.44
Desulfovibrio	94	4	0.94	0.32
Ruminococcus	98	4	0.92	0.20
Rhodocyclus	82	4	0.89	0.58
Pasteurella	92	4	0.84	0.18
Alistipes	65	4	0.70	0.42
Anaerofilum	59	4	0.61	0.34
Faecalibacterium	51	4	0.49	0.33
Mucispirillum	40	4	0.43	0.36
Oscillospira	33	4	0.32	0.39
Lactobacillus	33	4	0.32	0.22
Papillibacter	21	4	0.20	0.41

[1]Occurrence indicates the number of positive reads by a novel tag bacterial diversity amplification method (bTEFAP); the number of hens positive for each bacterial genus, and the proportion of the total microbial population represented by each genera are shown.

and *Megamonas* were found in both the fully fed and alfalfa crumble-fed groups, but not in the FW group.

DISCUSSION

Starvation causes stress in food animals and affects the intestinal microbial population (Gregory et al., 2000), including in laying hens (Durant et al., 1999; Holt, 2003). More than 75% of the laying hens in the United States are molted to induce a new egg-laying cycle, often by withdrawing feed for a period of more than 10 d (Bell, 2003). Starvation reduces the amount of substrate present in the gut for bacteria to ferment. This low nutrient concentration environment provides a selective pressure in the gut for bacteria that can survive long-term starvation (e.g., that can attach to epithelial tissues or utilize epithelial mucus or tissue sloughed into the gut, or both), thereby reducing the microbial diversity within the gut (Nettelbladt et al., 1997). In the present study, we found that hens undergoing FW had the fewest genera of bacteria present in their cecas, and hens molted by feeding alfalfa crumble had the greatest bacterial diversity among the 3 treatments. Of the 59 genera isolated from hens undergoing FW, only 12 genera (20.3%) were present in all 4 of the hens, compared with 23 of 64 genera (35.9%), and 30 of 78 genera (38.5%) being present in all hens from the full-feed and alfalfa crumble treatment groups, respectively. It appears, based on the present data, that cecal bacterial diversity is enhanced by feeding alfalfa crumble to hens during molting induction compared with simply using FW.

Cecal populations of *Salmonella* were not detected by traditional enrichment and plating in our study, yet *Salmonella* was the second most common genus in the cecas of the hens that underwent FW according to bTEFAP analysis based on partial ribosomal amplification followed by pyrosequencing (Dowd et al., 2008). These data agree with previously published reports indicating that FW during molting increased *Salmonella* populations in the gut of poultry (Holt, 1993, 2003). Because the diversity of the microbial population of the gut is reduced by FW, this could be a simple explanation for the increased incidence of *Salmonella* colonization of hens that have undergone FW.

Alfalfa has a relatively high fiber content and a long transit time in the gastrointestinal tract of poultry, but is indigestible by poultry, resulting in the bacterial fermentation of substrate to volatile fatty acids (**VFA**), which are toxic to some bacteria in the gut (Hollowell and Wolin, 1965). Consequently, VFA and other short-chain organic acids have been used as direct (as a feed additive) or indirect (as an end product of probiotic addition) techniques for reducing pathogen populations in the gut (Prohaszka and Baron, 1983). The lack of substrate in the gut of hens undergoing FW reduces VFA concentrations in the lower gut (Dunkley et al., 2007), which could be a contributing factor in the re-

duction of *Salmonella* in the birds fed a typical ration or alfalfa crumble.

Research has shown that feeding alfalfa meal crumble to laying hens is an effective way to induce molting that reduces animal welfare concerns related to FW as a method to induce a new egg production cycle (Landers et al., 2005). Although further evaluation of the effects of feeding alfalfa meal crumble in place of FW on egg production and subsequent feed efficiency is needed, our present study indicates that microbial diversity of the ceca is enhanced by feeding alfalfa crumble to molting hens. Data in this study support the concept that decreased microbial diversity of the gut can facilitate colonization of the gut by *Salmonella* by removing natural competitive barriers from the ecosystem. However, further studies using these molecular ecosystem analysis tools are obviously needed to more fully understand the normal gastrointestinal flora associated with the most efficient production, as well as the impact of changes made in the diet or management procedures of hens or broilers.

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